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connection with Application No. PQ 0848 for a patent by THE UNIVERSITY
OF MELBOURNE filed on 08 June 1999.

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Twenty-third day of June 2000

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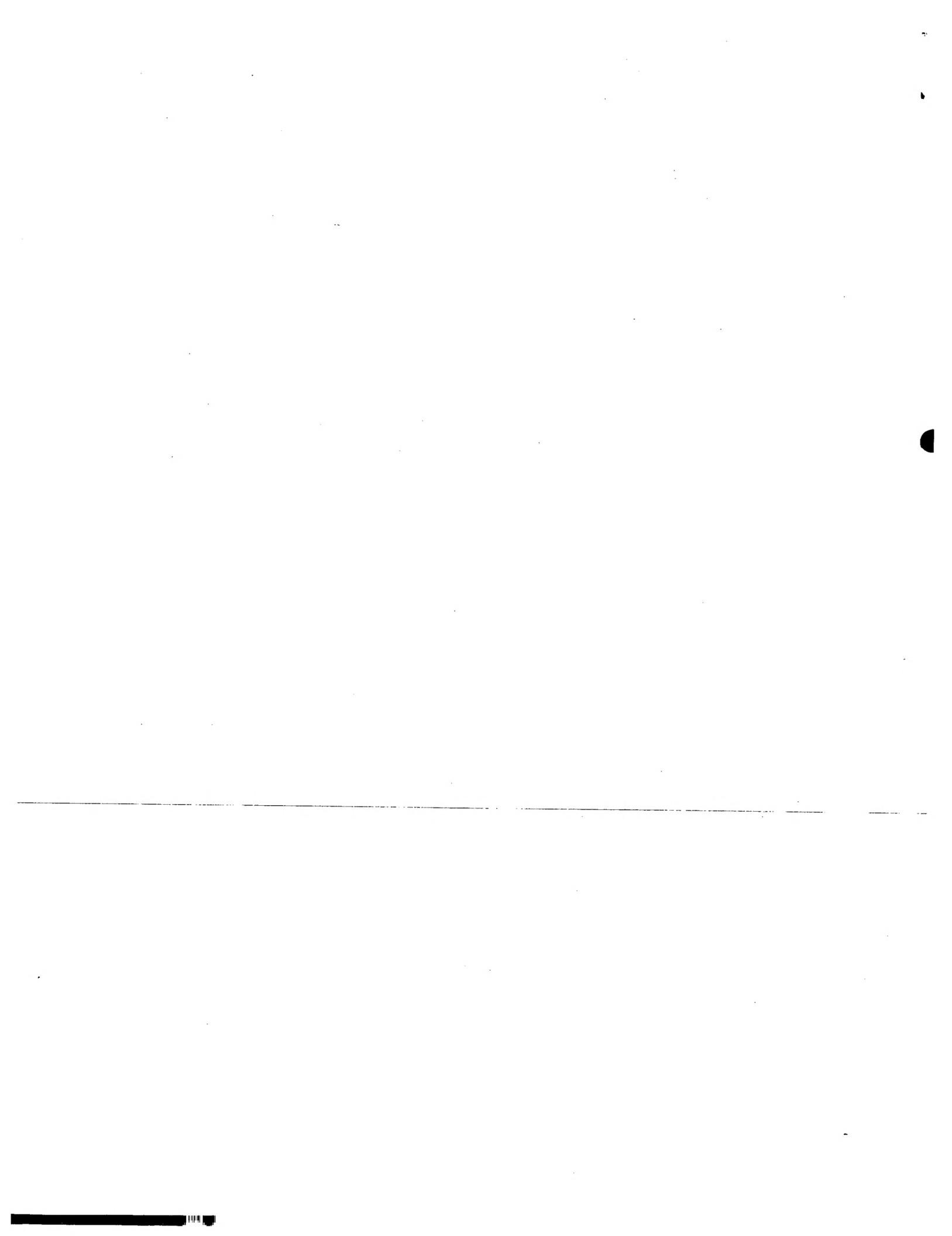
AUSTRALIA

Patents Act 1990

PROVISIONAL SPECIFICATION

Applicant(s):

THE UNIVERSITY OF MELBOURNE

Invention Title:

NEUROTROPHIN AGONISTS

The invention is described in the following statement:

NEUROTROPHIN AGONISTS

Field of the Invention

This invention relates to methods and compositions for promoting nerve cell growth, and in particular to agonists of brain-derived neurotrophic factor. The invention relates more particularly to agonists which are derivatives of peptides based on the structures of the solvent-exposed loops 2 and 4 of brain-derived neurotrophic factor.

Background of the Invention

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family of neurotrophic factors, which includes nerve growth factor (NGF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5) and neurotrophin-6 (Thoenen, 1991; Götz et al, 1994).

These factors promote the survival of neurons during embryonic development, and thus play a vital role in shaping the vertebrate nervous system. Between them, the neurotrophins support the survival of a wide range of peripheral and central neurons, although each individual neurotrophin acts on specific neuronal populations (for review see Thoenen, 1991; Götz et al, 1994).

In a variety of in vitro and in vivo models, BDNF has been shown to promote neuronal survival during embryonic development, and to prevent neuronal degeneration resulting from disease or injury. Furthermore, several BDNF-responsive neuronal populations have been implicated in human neurodegenerative disease. For example, in the central nervous system BDNF acts as a potent neurotrophic factor for cranial and spinal motor neurons which degenerate in amyotrophic lateral sclerosis (Thoenen et al, 1993), as well as for dopaminergic neurons of the substantia nigra which are lost in Parkinson's disease (Spina et al, 1992). In the periphery, BDNF has neurotrophic actions on small fibre sensory neurons

involved in several types of sensory neuropathy (Lindsay, 1994).

The biological effects of BDNF and the other neurotrophins are mediated by binding to two classes of cellular receptor: members of the trk family of receptor tyrosine kinases, and the low affinity neurotrophin receptor, p75. Specific neurotrophins bind with high affinity (K_d approximately 10^{-11} M) to particular trk members expressed by responsive neurons: thus NGF and NT-3 bind to trkA; BDNF and NT-4/5 bind to trkB; NT-3 binds to trkC. Binding of a neurotrophin to its specific trk receptor causes receptor homodimerisation, triggering the intrinsic kinase domains of the receptors to autophosphorylate intracellular tyrosine residues, and thus initiating signal transduction cascades leading to neuronal survival (Barbacid, 1994). In contrast, p75 acts as a common low affinity receptor for the neurotrophins, and binds each with comparable affinity (K_d approximately 10^{-9} M); p75 is expressed widely on central and peripheral neurons as well as on other cell types, such as Schwann cells (for review see Chao and Hempstead, 1995).

While the role of the trk members in signalling the neurotrophic effects of the neurotrophins is well established, the function of p75 remains controversial. Although there is compelling evidence that p75 either modulates responses mediated by trk members or itself plays a part in survival signalling, the final effect of p75 appears to depend on the relative levels of expression of p75 and trk (Kaplan and Miller, 1997). Of particular interest are the observations that p75 may, under certain circumstances, cause apoptosis either in the absence (Rabizadeh et al, 1993; Barrett and Bartlett, 1994) or presence (Frade et al, 1996) of bound neurotrophin. This "death signal" of p75 may be mediated by an intracellular region homologous to the death-signalling domains of tumour necrosis factor (TNF) receptor-1 and Fas (Chapman, 1995).

The neurotrophins are homodimers which consist of two identical protomers of approximately 120 amino acids, held together by hydrophobic interactions. The overall amino acid homology between the different neurotrophins is approximately 50%, and sequence alignment between the members reveals a common pattern of sequence homology and variability (Ibáñez et al, 1993). X-ray crystal structures have been determined for the NGF homodimer (McDonald et al, 1991) and for a BDNF/NT-3 heterodimer (Robinson et al, 1995), revealing a common fold for the neurotrophins. This structure is depicted in Figure 1. In the neurotrophin protomer, the regions of high sequence homology exist as seven β-strands, which contribute to three longitudinal antiparallel twisted β-sheets. This structure is locked by a "cysteine knot" of three disulphide bridges. The six cysteine residues which participate in the cysteine knot structure are fully conserved in all the neurotrophins. The three pairs of β-strands are linked by three β-hairpin loops (loop 1, loop 2 and loop 4) and a longer loop (loop 3), which correspond predominantly to the regions of sequence variability.

It has been postulated that the β-hairpin loop regions of the neurotrophins are responsible for the specificity of different trk receptors, and thus are important regions in receptor binding and activation. In general, structure-activity data obtained from the neurotrophins support this hypothesis. Site-directed mutagenesis studies have identified amino acid residues of in loop 2 of BDNF which are important for binding to trkB and for biological activity. Insertion of this region of BDNF into NGF gave a chimaeric protein which, unlike native NGF, bound to trkB and displayed BDNF-like biological activity (Ibáñez et al, 1993). Additional residues in loop 3 (Gln^{84}) and loop 4 (Lys^{96} and Arg^{97}) have been shown to be important in activation of trkB, but are thought not to be involved in receptor binding. When mapped on to the three-dimensional structure of BDNF, these residues are solvent-

accessible, and together form a binding surface that almost exclusively spans the top half of the molecule.

Other site-directed mutagenesis studies have shown that three positively-charged residues in each of the neurotrophins are of paramount importance in binding to p75 (Ibáñez et al, 1992; Rydén et al, 1995). These data are consistent with the idea that p75 shares a common binding interface with the neurotrophin family. There are, however, differences in the position of these three residues in different neurotrophins: in NGF, NT-3 and NT-4/5, the three positively-charged residues are spread across two adjacent loops, while in BDNF the three positively-charged residues are contiguous amino acids (Lys^{95}-Lys^{96}-Arg^{97}), located on loop 4 (Figure 1).

The ability of exogenously administered neurotrophic factors such as BDNF to rescue neurons in a variety of in vivo models of neurodegeneration has led to the widespread belief that neurotrophins and other neurotrophic factors offer exciting prospects for the treatment of neurodegenerative diseases, such as motor neuron disease and peripheral neuropathies (for review see Hefti, 1994). Unfortunately, because they are proteins, neurotrophic factors are orally inactive, are unable to cross the blood-brain barrier, and typically have a short half-life in plasma (Dittrich et al, 1994). Thus the recombinant human neurotrophic factors themselves are unlikely to be optimal agents for the long-term treatment of neurodegenerative disease. Indeed, the lack of success thus far of neurotrophic factors in clinical trials for the treatment of motor neuron disease has been attributed to the inability of the proteins to reach their targets in the central nervous system (CNS) following subcutaneous administration (Penn et al, 1997). One means of circumventing these problems would be to develop low molecular weight, non-peptidic analogues of neurotrophic factors with improved pharmacokinetic characteristics.

For example, Australian Patent Application No. 24264/97 by Regents of the University of California, and Longo et al, 1997, disclose low molecular weight compounds, 8-9 amino acids long and incorporating a D-penicillamine

5   group, which bind to the p75 region of the NGF receptor, and promote neuronal survival in primary cultures of chick dorsal root ganglia. The compounds form undefined (either parallel or anti-parallel) monocyclic dimers of 16-18 amino acids. International Patent Application

10   No. WO 95/15593 by Queen's University at Kingston discloses bicyclic peptides based on the cysteine knot region, which is distal from loops 1-4 of BDNF; these bicyclic peptides act as neurotrophin antagonists in the chick dorsal root ganglion assay. Cyclic peptides based on various loop

15   regions of NGF have been shown to interfere with NGF-mediated biological activity (LeSauter et al, 1995).

We have used a model of the three-dimensional structure of BDNF to design small, conformationally-constrained peptides that mimic the receptor-binding loops

20   of BDNF. These peptides have been synthesised and purified, and then assayed in cultures of embryonic chick sensory neurons, a subpopulation of which require BDNF for survival.

Monomeric cyclic peptides designed in this manner

25   from loop 2 — believed to be the major region of BDNF contributing to trkB binding and activation — act as specific antagonists of BDNF-mediated neuronal survival in cell culture. We have investigated structure-activity relationships in these peptides by conducting an alanine

30   scan, and have used pharmacodynamic simulations to model the anticipated competitive mode of antagonism of these peptides (O'Leary and Hughes, 1998).

We have now used the structure-activity data obtained for these monomeric cyclic peptides to design

35   bicyclic dimeric peptides with BDNF agonist activity. The peptides consist of two monocyclic peptides connected by a linking moiety. It is surprising that the linking moiety

in some of the dimers found to be active is incorporated at sites other than those predicted to be optimal on the basis of the structure-activity relationships. In addition, the linker distance found to give optimal activity was shorter
5 than that predicted from our design template.

By combining these data, we have been able to create a novel class of tricyclic dimeric peptides with markedly improved potency over the dimeric peptides.

In contrast, monomeric cyclic peptides based on
10 the p75-binding tripeptide sequence found in loop 4 had no inhibitory effects on the neuronal survival activity of either BDNF or NGF (Zwar and Hughes, 1997). To our surprise, however, when tested in the absence of neurotrophin one of these monomeric peptides acted as a
15 BDNF-like agonist, able to promote the survival of chick sensory neurons in culture.

Summary of the Invention

According to a first aspect, the invention
20 provides a cyclic compound of one or more cyclic moieties, which has a biological activity of brain-derived neurotrophic factor (BDNF).

In one embodiment, the compound is a bicyclic dimeric compound (that is, a compound composed of two
25 monocyclic compounds connected by a chemical linker) based on loop 2 of BDNF, of general formula:

```
          monomeric                  monomeric
30     loop 2 sequence—linker—loop 2 sequence
          └─constraint─┘          └─constraint─┘
```
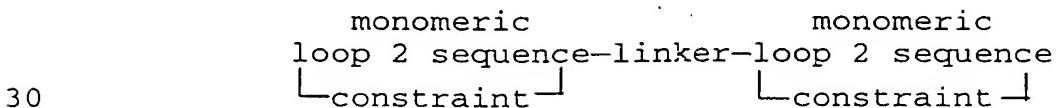
where:

"monomeric loop 2 sequence" represents a sequence of amino acid residues or functional equivalents thereof,
35 which is sustantially homologous to the loop 2 region of BDNF, and comprises all or part of the following sequence:

Glu^{40}-Lys^{41}-Val^{42}-Pro^{43}-Val^{44}-Ser^{45}-Lys^{46}-Gly^{47}-
Gln^{48}-Leu^{49}-Lys^{50}-Gln^{51};

"constraint" represents a conformational
constraint imposed on the monomeric loop 2 sequence, which
acts to limit the flexibility of the monomeric loop 2
sequence, for example by covalently linking all or part of
the monomeric loop 2 sequence to form a cyclic structure
(ring); and linker represents any chemically and
biologically compatible covalent grouping of atoms serving
to link two monomeric loop 2 sequences and their associated
constraints to give a dimeric, bicyclic peptide.

Generally, the preferred linking groups have 0 to
20 carbon atoms, and 0 to 10 heteroatoms (N, O, S, P etc.),
and may be straight chain or branched, may contain
saturated, unsaturated and/or aromatic rings, may contain
single and/or double bonds, and may contain chemical groups
such as amide, ester, disulphide, thioether, ether,
phosphate, amine·and the like.

The "constraint" can be obtained by several
methods, including but not limited to:

(i) cyclising the N-terminal amine with the C-
terminal carboxyl acid function, either directly via an
amide bond between the N-terminal nitrogen and C-terminal
carbonyl, or indirectly via a spacer group, for example by
condensation with an ω-amino carboxylic acid;

(ii) cyclising via the formation of a covalent
bond between the side chains of two residues, such as an
amide bond between between a lysine residue and either an
aspartic acid or glutamic acid residue, or a disulphide
bond between two cysteine residues, or a thioether bond
between a cysteine residue and an ω-halogenated amino acid
residue, either directly or via a spacer group as described
in (i) above. The residues contributing the side chains
may be derived from the monomeric loop 2 sequence itself,
or may be incorporated into or added on to the monomeric
loop 2 sequence for this purpose; and,

(iii) cyclising via the formation of an amide bond between a side chain (for example of a lysine or aspartate residue) and either the C-terminal carboxyl or N-terminal amine, either directly or a spacer group as
5   described in (i) above. The residues contributing the side chains may be derived from the monomeric loop 2 sequence itself, or may be incorporated into or added on to the monomeric loop 2 sequence for this purpose.

In a second embodiment, the compound is a
10 tricyclic dimeric compound (that is, a compound composed of two monocyclic compounds connected by two chemical linkers) based on loop 2 of BDNF of general formula:

```
        monomeric——linker1——monomeric
15      loop 2 sequence——linker2——loop 2 sequence
        └─constraint─┘            └─constraint─┘
```
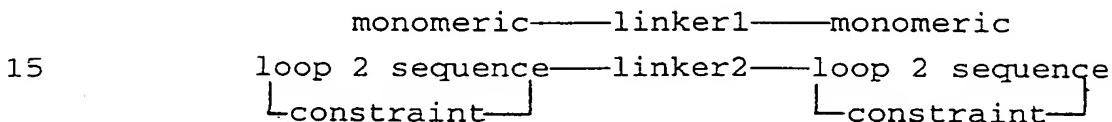
where:

"monomeric loop 2 sequence" is a sequence of
20 amino acid residues or functional equivalents thereof, which is substantially homologous to the loop 2 region of BDNF, and comprises all or part of the following sequence:
Glu^{40}-Lys^{41}-Val^{42}-Pro^{43}-Val^{44}-Ser^{45}-Lys^{46}-Gly^{47}-Gln^{48}-Leu^{49}-Lys^{50}-Gln^{51};
25 "constraint" represents a conformational constraint imposed on the monomeric loop 2 sequence, which acts to limit the flexibility of the monomeric loop 2 sequence, for example by covalently linking all or part of the monomeric loop 2 sequence to form a cyclic structure
30 (ring); and "linker1" and "linker2" represent any chemically and biologically compatible covalent grouping of atom serving to link two monomeric loop 2 sequences and their associated constraints to give a dimeric, tricyclic peptide. These linkers may be the same or different
35   Generally, the preferred linking groups have 0 to 20 carbon atoms, and 0 to 10 heteroatoms (N, O, S, P etc.), and may be straight or branched, may contain saturated,
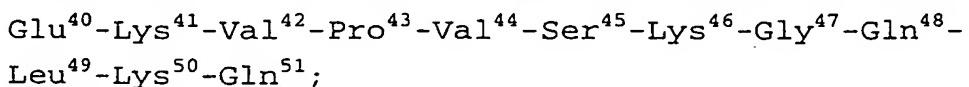
unsaturated and/or aromatic rings, may contain single and/or double bonds, and may contain chemical groups such as amide, ester, disulphide, thioether, ether, phosphate, amine and the like.

The "constraint" can be obtained by several methods, including but not limited to:

(i) cyclising the N-terminal amine with the C-terminal carboxyl acid function either directly via an amide bond between the N-terminal nitrogen and C-terminal carbonyl, or indirectly via a spacer group, for example by condensation with an ω-amino carboxylic acid;

(ii) cyclising via the formation of a covalent bond between the side chains of two residues, such as an amide bond between between a lysine residue and either an aspartic acid or glutamic acid residue, or a disulphide bond between two cysteine residues, or a thioether bond between a cysteine residue and an ω-halogenated amino acid residue, either directly or via a spacer group as described in (i) above. The residues contributing the side chains may be derived from the "monomeric loop 2 sequence" itself, or may be incorporated into or added onto the monomeric loop 2 sequence for this purpose; and,

(iii) cyclising via the formation of an amide bond between a side chain (for example of a lysine or aspartate residue) and either the C-terminal carboxyl or N-terminal amine, either directly or via the intermediacy of a spacer group as described in (i) above. The residues contributing the side chains may be derived from the monomeric loop 2 sequence itself, or may be incorporated into or added onto the monomeric loop 2 sequence for this purpose.

In third embodiment, the compound is a monomeric, monocyclic compound based on the p75 binding region of loop 4 of BDNF and incorporating a molecular spacer of the general formula:

$$
\underbrace{\text{monomeric} \atop \text{loop 4 sequence}}_{\text{constraint'}}
$$

5   where:

monomeric loop 4 sequence represents a sequence of amino acid residues or functional equivalents thereof, which is sustantially homologous to the p75 binding region of loop 4 of BDNF, and comprises all or part of the

10   following sequence:

$Lys^{95}-Lys^{96}-Arg^{97}$; and,

"constraint'" represents a conformational constraint imposed on the monomeric loop 4 sequence, which acts to limit the flexibility of the "monomeric loop 4

15   sequence", for example by covalently linking all or part of the "monomeric loop 4 sequence" to form a cyclic structure (ring).

The "constraint'" can be derived by several methods, including but not limited to:

20   (i) cyclising the N-terminal amine with the C-terminal carboxyl acid function, either directly via an amide bond between the N-terminal nitrogen and C-terminal carbonyl, or indirectly via a spacer group, such as one or more additional amino acid resdues, including α- and ω-

25   amino carboxylic acid residues;

(ii) cyclising via the formation of a covalent bond between the side chains of two residues, such as an amide bond between between a lysine residue and either an aspartic acid or glutamic acid residue, or a disulphide

30   bond between two cysteine residues, or a thioether bond between a cysteine residue and an ω-halogenated amino acid residue, either directly or via a spacer group as described in (i) above. The residues contributing the side chains may be derived from the "monomeric loop 4 sequence" itself,

35   or may be incorporated into or added on to the "monomeric loop 4 sequence" for this purpose; and

(iii) cyclising via the formation of an amide bond between a side chain (for example of a lysine or aspartate residue) and either the C-terminal carboxyl or N-terminal amine, either directly or via a spacer group as described in (i) above. The residues contributing the side chains may be derived from the monomeric loop 4 sequence itself, or may be incorporated into or added on to the monomeric loop 4 sequence for this purpose.

Sequences encompassing conservative substitutions of amino acids are within the scope of the invention, provided that the biological activity is retained.

It is to be clearly understood that the compounds of the invention include peptide analogues, including but not limited to the following:

1. Compounds in which one or more amino acids is replaced by its corresponding D-amino acid. The skilled person will be aware that retro-inverso amino acid sequences can be synthesised by standard methods; see for example Chorev and Goodman, 1993;

2. Peptidomimetic compounds, in which the peptide bond is replaced by a structure more resistant to metabolic degradation. See for example Olson et al, 1993.

3. Compounds in which individual amino acids are replaced by analogous structures, for example gem-diaminoalkyl groups or alkylmalonyl groups, with or without modified termini or alkyl, acyl or amine substitutions to modify their charge.

The use of such alternative structures can provide significantly longer half-life in the body, since they are more resistant to breakdown under physiological conditions.

Methods for combinatorial synthesis of peptide analogues and for screening of peptides and peptide analogues are well known in the art (see for example Gallop et al, 1994). It is particularly contemplated that the compounds of the invention are useful as templates for

design and synthesis of compounds of improved activity, stability and bioavailability.

Preferably where amino acid substitution is used, the substitution is conservative, *i.e.* an amino acid is

5   replaced by one of similar size and with similar charge properties.

In particularly preferred embodiments the bicyclic dimer is:

10    $(L2-8P2C)_2$

```
       ┌─────────────────┐              ┌─────────────────┐
C-V-C-V-S-K-G-Q-L-C        C-V-C-V-S-K-G-Q-L-C
  └─────────────────┘        └─────────────────┘
```

or

15

$(L2-8S4C)_2$

```
        ┌─────────────┐              ┌─────────────┐
C-V-P-V-C-K-G-Q-L-C        C-V-P-V-C-K-G-Q-L-C
  └─────────────────┘        └─────────────────┘
```
,
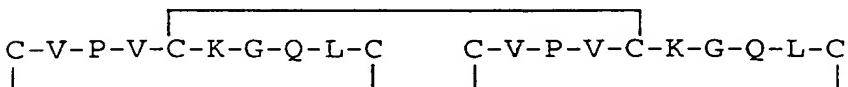
20   where the dimeric bicyclic peptides $(L2-8P2C)_2$ and $(L2-8S4C)_2$ consist of monomeric loop 2 sequences constrained by disulphide bonds formed between cysteine residues added to the loop 2 sequence and joined by a linker consisting of a disulphide bond formed between

25   cysteine residues substituted into the loop 2 sequence, or

$(L2-8\&E+K)_2$

```
                        ┌──────────────────────────────┐
Ac-C-V-P-V-S-K-G-Q-L-C-E-NH2    Ac-C-V-P-V-S-K-G-Q-L-C-K-NH2
     └─────────────────┘            └─────────────────┘
```

30   where the dimeric bicyclic peptide $(L2-8\&E+K)_2$ consists of monomeric loop 2 sequences constrained by disulphide bonds formed between cysteine residues added to the loop 2 sequence and joined by a linker consisting of an

35   amide bond formed between a glutamate and a lysine residue added to the loop 2 sequence;

the tricyclic dimer is:

(L2-8S4C&E+K)₂

```
        ┌─────────────────────────┐         ┌───────────────────┐
  5  Ac-C-V-P-V-C-K-G-Q-L-C-E-NH₂     Ac-C-V-P-V-C-K-G-Q-L-C-K-NH₂
        └──────────────────────┘    └─┘         └─────────────┘
                                  └──────────────────────────────┘
```
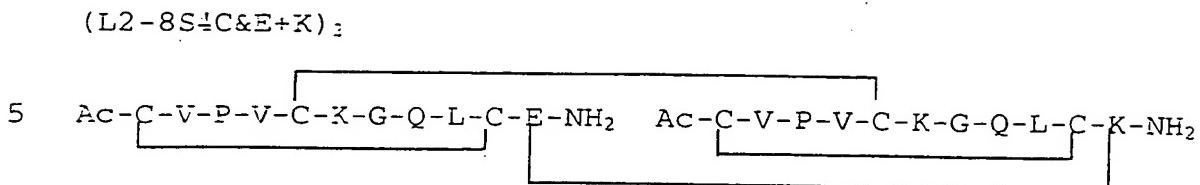
where the dimeric tricyclic peptide (L2-8S4C&E+K)₂ consists of monomeric loop 2 sequences constrained by disulphide bonds formed between cysteine residues added to the loop 2 sequence, and joined by one linker consisting of a disulphide bond formed between cysteine residues substituted into the loop 2 sequence and a second substituted consisting of an amide bond formed between a glutamate and a lysine residue added to the loop 2 sequence, and

the monomeric, monocyclic compound is

                    L4-3pA
```
                  ┌─pAKKR─┐
                  └───────┘
```

where p is a D-proline residue, and the monomeric cyclic compound L4-3pA is cyclised by condensing its amino- and carboxyl-termini directly via an amide bond.

The invention further provides a pharmaceutical composition, comprising a compound according to the invention together with a pharmaceutically-acceptable carrier.

The composition may be formulated so as to be suitable for a variety of routes of administration, for example intravenous, subcutaneous, intramuscular or intrathecal or intraventricular injection, or for topical administration.

The exact formulation will depend on the individual route of administration. Methods and pharmaceutical carriers for preparation of pharmaceutical

compositions are well known in the art, as set out in textbooks such as Remington's Pharmaceutical Sciences, 17th Edition, Mack Publishing Company, Easton, Pennsylvania, USA. Pharmaceutically-acceptable carriers include

5 conventional carriers which are suitable for use with peptide-based drugs, including diluents, excipients, and preservatives and the like. For example, carriers such as dextrose, mannitol, sucrose, or lactose, buffer systems such as acetate, citrate and phosphate, and bulking agents

10 such as serum albumin, preferably human serum albumin, may be used.

The invention also provides a culture medium additive for promotion of growth of neuronal cells *in vitro*, comprising a compound according to the invention

15 together with a carrier or diluent which does not adversely effect the growth of cells in culture. Suitable carriers and diluents will be well known to the person skilled in art, and include physiologically acceptable fluids such as water, saline solution, or buffer solutions.

20 The optimal concentration of compound will vary according to the cell type and the culture conditions, but will generally be in the range 1-500µM, preferably 1-100µM.

The invention further provides a method of treatment of a condition characterised by neuronal deficit

25 or neuronal death, comprising the step of administering an effective amount of a compound of the invention to a subject in need of such treatment.

It is contemplated that the method of the invention is suitable for treatment of conditions including

30 but not limited to neurodegenerative diseases such as motor neurone disease (amyotrophic lateral sclerosis), progressive spinal muscular atrophy, infantile muscular atrophy, Charcot-Marie-Tooth disease, Parkinson's Disease, Parkinson-Plus syndrome, Guamanian Parkinsonian dementia

35 complex, progressive bulbar atrophy, Alzheimer's disease and the like, other neurodegenerative conditions such as those arising from ischaemia, hypoxia, neural injury,

surgery, exposure to neurotoxins such as N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), and peripheral sensory neuropathies, including those resulting from exposure to drugs (such as cis-platin) and toxins and resulting from diabetes, for example mononeuropathy multiplex.

The dose required will depend on the nature and severity of the condition to be treated, and the route of administration, and will be at the discretion of the attending physician or surgeon. A suitable route, frequency of administration, and dosage can readily be established using conventional clinical trial methodology.

Throughout this specifciation, the amino acid numbering is the same as in mature BDNF, and conventional single-letter amino acid code is used.

For the purposes of this specification it will be clearly understood that the word "comprising" means "including but not limited to", and that the word "comprises" has a corresponding meaning.

The term biological activity with reference to BDNF means a biological activity which is normally promoted, either directly or indirectly, by the presence of BDNF, and includes, but is not limited to, BDNF binding to the p75 receptor or the trkB receptor, neuron survival, neuron differentiation, including neuron process formation and neurite outgrowth, and biochemical effects such as induction of enzymes which are stimulated by BDNF. Such biological activities can be measured by conventional *in vitro* and *in vivo* assays, such as the chick dorsal root ganglion assay described herein by Barde *et al* (1980) and the neurite outgrowth, and *in vivo* kindling assays described in WO 97/15593 and by Riopelle *et al* (1982).

It will be clearly understood that the compounds of the invention may be synthesised by any suitable method. Solid phase methods such as those developed for synthesis of peptides and peptidomimetic compounds are preferred, including but not limited to the Fmoc solid phase peptide synthesis method described herein, the Boc solid phase

peptide synthesis method, and PIN synthesis methods (for review, see Maeji *et al.*, 1995). Those skilled in the art will readily be able to select the most suitable method for any given compound of the invention.

Brief Description of the Figures

Figure 1   shows the backbone trace of the three-dimensional structure of BDNF dimer (one monomer in black, the other grey), showing the positions of the loop 2 (trkB binding) and loop 4 (predominantly p75 binding) regions. Sidechains of the p75 binding tripeptide in loop 4 (Lys-Lys-Arg) are shown.

Figure 2 illustrates the molecular modelling of monomeric cyclic loop 2 analogues. An α-carbon to α-carbon trace of the native loop 2 of BDNF is shown, superimposed with low-energy conformations of loop 2 analogues L2-12, L2-10, L2-8 and L2-6, each of which is constrained by a disulphide bridge (indicated by arrows).

Figure 3 shows the concentration-response curves of monomeric cyclic loop 2 analogues in competition with BDNF. The monomeric cyclic loop 2 analogues L2-12 (closed triangles), L2-12a (open triangles), L2-10 (open squares), L2-8 (closed diamonds) and L2-6 (open diamonds) and the monomeric linear peptide L2-12b (closed squares) were assayed in competition with BDNF (4×10^{-11} M) in cultures of E8 to E10 chick sensory neurons. Surviving neurons were counted after 48 hrs in culture, and these counts were then expressed as a percentage of originally-plated viable neurons and normalised such that survival in cultures containing BDNF alone (P; positive control; closed circle) was set to 100% and survival in cultures with neither BDNF nor loop 2 analogue (N; negative control; open circle) to 0%. Data are expressed as the mean ± SEM from at least 8 observations (n=8) from 4 independent experiments.

Figure 4 shows the concentration-response curves of monomeric cyclic peptide L2-12, alone and in competition with NGF. Surviving neurons were counted after 48 hrs in

culture, and these counts expressed as a percentage of originally-plated viable neurons and normalised such that survival in cultures containing NGF alone (P; positive control; closed circle) was set to 100% and survival in
5   cultures with neither NGF nor L2-12 (N; negative control; open circle) to 0%. When assayed in the absence of NGF, L2-12 (closed triangles) produced no significant effect on neuronal survival when compared to survival in negative control cultures ($p > 0.05$, ANOVA; n=6). L2-12 (open
10   triangles) assayed in competition with NGF (4×10^{-11} M) produced no significant effect on NGF-mediated survival ($p > 0.05$, ANOVA; n=6). Data are expressed as the mean ± SEM.

Figure 5 shows the effect of a monomeric cyclic loop 2 analogue (L2-12a) on the concentration-response
15   curve of BDNF. L2-12a (1×10^{-7} M) was assayed in competition with BDNF (1.8×10^{-13} to 1.8×10^{-10} M, 0.51 og increments) in cultures of E8-E10 sensory neurons. Surviving neurons were counted after 48 hrs in culture; these counts were expressed as a percentage of the number
20   of originally-plated viable neurons, and logistic sigmoidal curves fitted to the data. Compared to the BDNF control curve (closed circles), the BDNF curve in the presence of L2-12a (open circles) shows a significant depression in maximum (40%; ** $p < 0.005$, Student's unpaired two-tailed
25   t-test) and a 1.6 fold shift in pEC_{50}, although the latter is insignificant ($p > 0.05$, Student's unpaired two-tailed t-test). N (closed square) refers to negative control cultures in the absence of both BDNF and L2-12a. Data are expressed as the mean ± SEM of 8 observations (n=8) from 4
30   independent experiments.

Figure 6 illustrate the maximal inhibition of BDNF-mediated survival of cultured sensory neurons by monomeric cyclic loop 2 analogues systematically substituted with alanine. Loop 2 analogues were assayed in
35   competition with BDNF (4×10^{-11} M) in cultures of E8-E10 sensory neurons. Surviving neurons were counted after 48 hrs in culture, and these counts were expressed as a

percentage of originally-plated viable neurons then
normalised such that survival of cultures containing BDNF
was set as 100%, while that for cultures with neither BDNF
nor loop 2 analogue was set to 0%. Maximal inhibition of
5   BDNF-mediated survival was calculated by subtracting the
lowest value for BDNF-mediated survival from that of BDNF
alone (100%). Note that alanine substitution in the L2-12
sequence can affect the ability of these peptides to
modulate BDNF-mediated survival. Significant reduction in
10 inhibitory activity, compared to L2-12 (closed bar), was
observed when Ala was substituted for Val^3, Val^5, Ser^6
(***$p < 0.001$; ANOVA Bonferroni multiple comparisons test
n=12), Lys^{11} (*$p < 0.05$; n=12) and Gln^{12} (**$p < 0.01$; n=10).
No data (ND) were obtained for L2-12P4$\triangle$A. Data are
15 expressed as the mean $\pm$ SEM.

Figure 7A shows a schematic view of the two loop
two regions in the model of the three-dimensional structure
of the BDNF dimer, showing the interatomic distances (Å)
between α-carbon atoms of selected residues.

20   Figure 7B shows a schematic view of the
disulphide bridge of the cystine residue, showing the
average interatomic distance and 90% confidence interval
(90% CI) of α-carbon atoms, determined by conformational
analysis.

25   Figure 8 shows a graph of the survival of sensory
neurons in the presence of the bicyclic dimeric peptides
$(L2-8P2C)_2$, $(L2-8V3C)_2$ and $(L2-8S4C)_2$. Neurons were
prepared from chick dorsal root ganglia from embryonic
chicks (E8-E10), and surviving neurons counted after 48
30 hours in culture. Data are presented as a percentage of
the number of cells supported by BDNF (1ng/ml; 100%) after
the same period in culture. Survival in negative control
cultures was set to 0%. Highly significant differences in
neuronal survival in the presence of $(L2-8P2C)_2$, and (L2-
35 $8S4C)_2$ were observed compared to survival in negative
controls (ANOVA, *** p<0.001, Bonferroni multiple
comparisons test).

Figure 9 shows a graph of the survival of sensory neurons in the presence of the monomeric cyclic peptides L2-8P2C(Acm) and L2-8S4C(Acm). Neurons were prepared from dorsal root ganglia from embryonic chicks (E8-E10), and

5   surviving neurons counted after 48 hours in culture. Data are presented as a percentage of the number of cells supported by BDNF (1ng/ml; 100%) after the same period in culture. Survival in negative control cultures was set to 0%. Data were obtained from at least two independent

10   experiments.

Figure 10 shows a graph of the survival of sensory neurons in the presence of the amide-linked dimeric bicyclic peptide (L2-8&E+K)$_2$. Neurons were prepared from chick dorsal root ganglia obtained from embryonic chicks

15   (E8-E10), and surviving neurons counted after 48 hours in culture. Data are expressed as a percentage of the number of cells supported by BDNF (1ng/ml; 100%) after the same period in culture. Survival in negative control cultures was set to 0%. A highly significant difference in neuronal

20   survival in the presence of (L2-8&E+K)$_2$ was observed compared to survival in negative controls (ANOVA, $p<0.001$, Bonferroni multiple comparisons test).

Figure 11 shows a graph of the survival of sensory neurons in the presence of the dimeric tricyclic

25   loop 2 analogue (L2-8S4C&E+K)$_2$. Neurons were prepared from chick dorsal root ganglia obtained from embryonic chicks (E8-E10), and surviving neurons counted after 48 hours in culture. Data are expressed as a percentage of the number of cells supported by BDNF (1ng/ml; 100%) after the same

30   period in culture. Survival in negative control cultures was set to 0%. A highly significant difference in neuronal survival in the presence of (L2-8S4C&E+K)$_2$ was observed compared to survival in negative controls (ANOVA, $p<0.001$, Bonferroni multiple comparisons test).

35   Figure 12 shows a graph of the survival of sensory neurons in the presence of the monomeric cyclic loop 4-derived peptide L4-3pA(II) (closed circles).

Neurons were prepared from dorsal root ganglia from embryonic chicks (E8-E10) and surviving neurons counted after 48 hours in culture. B: positive control (BDNF 1 ng/ml); N: negative control (no peptide). ***

5   Significantly different to negative control (ANOVA, p < 0.001, Bonferroni mutiple comparisons test, n = 12).

Figure 13 shows a graph of the survival of sensory neurons in the presence of the monomeric cyclic loop 4-derived peptides L4-3pA(I), L4-3pA(II) and L4-3Hx,

10   and their linear homologues L4-3pAa and L4-3Hxa. All peptides were added at a concentration of 10^{-6} M. BDNF was added at 1 ng/ml. Neg: shows the survival in negative control cultures containing neither BDNF nor peptide. *** Significantly different to negative control (ANOVA, p <

15   0.001, Bonferroni mutiple comparisons test, n = 12).

Figure 14 shows a graph of the effect of the monomeric cyclic loop 4-derived peptides L4-3pA(I) (open diamonds), L4-3pA(II) (open squares) and L4-3Hx (open triangles), and their linear homologues L4-3pAa (crosses)

20   and L4-3Hxa (asterisks) on the neuronal survival effect mediated by BDNF (1 ng/ml). Over the concentration range tested (10^{-11} to 10^{-5}), none of the peptides exhibited a significant effect on BDNF mediated neuronal survival.

Figure 15 shows shows a graph of the effect of

25   the monomeric cyclic loop 4-derived peptides L4-3pA(I) (open diamonds), L4-3pA(II) (open squares) and L4-3Hx (open triangles), and their linear homologues L4-3pAa (crosses) and L4-3Hxa (asterisks) on the neuronal survival effect mediated by NGF (1 ng/ml). Over the concentration range

30   tested (10^{-11} to 10^{-5}), none of the peptides exhibited a significant effect on NGF mediated neuronal survival.

Figure 16 shows a graph of the survival of sensory neurons in the presence of the monomeric cyclic loop 4 peptides, L4-3Ap(I), L4-3Ap(II), L4-3AP(I) and L4-

35   3AP(II). All peptides were added at a concentration of 10^{-6} M. BDNF was added at a concentration of 1ng/ml. Neg shows the survival of control cultures containing neither

BDNF nor peptide. None of the peptides exhibited an effect on neuronal survival that was significantly different to that seen in negative control cultures.

5 Figure 17 shows a graph of the survival of sensory neurons in the presence of the monomeric cyclic loop 4 peptides L4-3K3ApA and L4-3K4ApA. The peptides were added at a concentration of 10^{-5} M. BDNF was added at a concentration of 1ng/ml. Neg shows the survival of control cultures containing neither BDNF nor peptide. Neither of

10 the peptides exhibited an effect on neuronal survival that was significantly different to that seen in negative control cultures.

Figure 18 shows the solution structure of peptide L4-3pA(II) derived by nuclear magnetic resonance (NMR)

15 spectroscopy. Depicted is an overlay of the twenty conformations of peptide L4-3pA(II) with the lowest target function in the software package DYANA, following 10000 steps of simulated annealing followed by 2000 steps on minimisation using the NMR-derived distance and dihedral

20 angle constraints. Residues are labelled and numbered.

DETAILED DESCRIPTION OF THE INVENTION

The invention will now be described by way of reference only to the non-limiting examples, and to the

25 Figures.

Abbreviations

BDNF	brain-derived neurotrophic factor	
E	embryonic day	
30	NGF	nerve growth factor
	NT-3	neurotrophin-3
	NT-4	neurotrophin-4
	TFA	trifluoroacetic acid
	NMR	nuclear magnetic resonance spectroscopy.

35

Materials

Mouse recombinant BDNF was a kind gift from Dr R Kolbeck and Professor Y-A Barde (Max-Planck-Institute for Psychiatry, Martinsried, Federal Republic of Germany).

5   NGF, purified from male mouse submaxillary gland was purchased from Boehringer-Mannheim (Mannheim, Federal Republic of Germany). Fertilised chicken eggs were obtained from Research Poultry Farms (Research, Victoria, Australia), trypsin from Worthington (Freehold, NJ,

10 U.S.A.), L-15 from GIBCO BRL (Grand Island, NY, U.S.A.), horse serum from CSL (Parkville, Victoria, Australia), Nunclon 10 cm tissue culture dishes from Nalge Nunc International (Roskilde, Denmark), Falcon Multiwell 48-well tissue culture plates from Becton Dickinson (Franklin

15 Lakes, NJ, U.S.A.) and mouse laminin, isolated from Englebreth-Holm-Swarm tumour cells, from Collaborative Biomedical Products (Bedford, MA, U.S.A.). Fmoc-amino acids and Wang resin were purchased from Auspep (Parkville, Victoria, Australia), PR-500 resin from Calbiochem-

20 NovaBiochem (Alexandria, New South Wales, Australia) and Econosil irregular packed HPLC columns from Alltech Associates (Baulkham Hills, New South Wales, Australia). Other reagents were purchased from Sigma (Castle Hill, New South Wales, Australia).

25

Example 1   Homology Modelling of BDNF

A model of the three-dimensional structure of murine BDNF was obtained by protein homology modelling techniques from murine NGF. This was performed by the

30 Swiss-Model automated protein homology server running at the Glaxo Institute for Molecular Biology in Geneva, Switzerland, accessed via the Internet (http://expasy.hcuge.ch/swissmod/SWISS-MODEL.html, Peitsch, 1995). Briefly, a three-dimensional model of the target

35 sequence is produced in the following manner: Swiss-Model searches the Brookhaven Protein Data Bank for the sequences of homologous proteins of known three-dimensional

structure.   Once a template sequence is found, Swiss-Model
produces a structural framework for the target sequence,
using a combination of sequence alignment tools and three-
dimensional superimposition.   Homologous regions of the
5   template protein form the structural backbone of the target
sequence, while non-conserved regions are built using the
three-dimensional structures of related sequences in the
Brookhaven Protein Data Bank.   Side chains not present on
the template protein are inserted, and all side chains are
10   corrected using a library of allowed rotamers.   The model
is then optimised by energy minimisation using the CHARMm
forcefield.

The co-ordinates of the BDNF monomer were
downloaded and the BDNF dimer constructed by superimposing
15   two monomers over the coordinates of selected conserved α-
carbon atoms in the NGF dimer, using the PC-based molecular
modelling software Hyperchem version 4.0 (Hypercube,
Ontario, Canada).

As expected, the model of the three-dimensional
20   structure of BDNF obtained by homology modelling possessed
an overall fold very similar to that of NGF, as shown in
Figure 2.   The validity of this structure was further
confirmed by its similarity to the crystal structure of a
BDNF/NT-3 heterodimer (Robinson *et al*, 1995), whose co-
25   ordinates were released some time after this work
commenced.

Example 2   Molecular Modelling of Monomeric Cyclic Loop
               2 Analogues
30   The molecular modelling of peptide analogues was
carried out using Hyperchem, as follows: After visual
inspection of the model obtained by homology modelling,
loop 2 was defined and excised from the three-dimensional
structure of BDNF, and various means of constraining
35   peptides to the native loop 2 conformation were
investigated.   Each constraint was built between the
terminal residues of the peptide, and these residues

geometrically optimised using the Polak-Ribiere algorithm
and MM+ forcefield to a local low-energy conformation.
These modelled peptides were assessed for their ability to
mimic the native conformation by measuring the root mean
square deviation of the peptide backbone to that of the
native loop following least squares superimposition.

From this model of BDNF the second β-hairpin loop
(loop 2) was defined as Glu^{40}-Lys^{41}-Val^{42}-Pro^{43}-Val^{44}-Ser^{45}-
Lys^{46}-Gly^{47}-Gln^{48}-Leu^{49}-Lys^{50}-Gln^{51}, where the amino acid
numbering, is the same as in mature BDNF. Peptide
analogues of this loop were modelled to investigate

(i)   what type of constraint would be most
appropriate to allow the peptides to mimic loop 2 in its
native loop conformation, and

(ii)   where in the sequence this constraint would
be best positioned.

As a result of these studies four peptides,
L2-12, L2-10, L2-8 and L2-6, each constrained by a
disulphide bridge between terminal cysteine residues, were
chosen for synthesis and biological examination. The sites
from which these peptides are derived are illustrated in
Figure 2.

Example 3   Synthesis of Monomeric Cyclic Loop 2 Peptides

Linear peptides in the free acid form were
assembled manually from Fmoc-amino acids on Wang resin
using batch-type solid phase methods (Fields and Noble,
1990). Both coupling and deprotection reactions were
assessed with the trinitrobenzenesulphonic acid test
(Thompson et al, 1995). The linear peptide amide L2-12a
was synthesised using continuous flow methods on PR-500
resin; coupling and deprotection steps were monitored
spectrophotometrically. Cleavage of peptides from the
resin and sidechain removal was accomplished with
trifluoroacetic acid (TFA)/ethanedithiol/H_2O (18:1:1).

Crude peptide products were analysed and purified
by reversed phase HPLC over Econosil C-18 irregular packed

columns. Gradients were tailored to individual runs, using combinations of solution A (0.1% TFA in H_2O) and solution B (0.1% TFA in 70% acetonitrile, 30% H_2O).

Purified peptide products were cyclised by
5   oxidising terminal cysteine residues to the disulphide in the presence of 10% dimethylsulphoxide in 0.1M NH_4HCO_3 solution at pH 8.0 (Tam *et al*, 1991). Reactions were monitored and the cyclised products purified by high performance liquid chromatography. The purity of the
10   peptides was further assessed by capillary zone electrophoresis (Applied Biosystems 270A). The identity of each peptide was confirmed by mass spectrometry using either electrospray (Micromass platform II with electrospray source) or fast atom bombardment (Jeol JMS-Dx
15   300) techniques.

All the peptide analogues were synthesised with free amino and carboxyl termini, except L2-12a, which had acetylated amino and amidated carboxyl termini. The linear peptide L2-12b was synthesised without terminal Cys
20   residues, to ensure that it remained in a linear form during biological assays. The peptides L2-12E1ΔA to L2-12Q12ΔA contain alanine substitution at the indicated positions in the L2-12 sequence.

The peptide analogues synthesised are listed in
25   Table 1.

Table 1

Monomeric Cyclic Loop 2 Analogue Sequences

Code	Peptide analogue sequence
L2-12	C-E-K-V-P-V-S-K-G-Q-L-K-Q-C
L2-12a	Ac-C-E-K-V-P-V-S-K-G-Q-L-K-Q-C-NH$_2$
L2-12b	E-K-V-P-V-S-K-G-Q-L-K-Q
L2-10	C-K-V-P-V-S-K-G-Q-L-K-C
L2-8	C-V-P-V-S-K-G-Q-L-C
L2-6	C-P-V-S-K-G-Q-C
L2-12E1ΔA	C-**A**-K-V-P-V-S-K-G-Q-L-K-Q-C
L2-12K2ΔA	C-E-**A**-V-P-V-S-K-G-Q-L-K-Q-C
L2-12V3ΔA	C-E-K-**A**-P-V-S-K-G-Q-L-K-Q-C
L2-12P4ΔA[a]	C-E-K-V-**A**-V-S-K-G-Q-L-K-Q-C
L2-12V5ΔA	C-E-K-V-P-**A**-S-K-G-Q-L-K-Q-C
L2-12S6ΔA	C-E-K-V-P-V-**A**-K-G-Q-L-K-Q-C
L2-12K7ΔA	C-E-K-V-P-V-S-**A**-G-Q-L-K-Q-C
L2-12G8ΔA	C-E-K-V-P-V-S-K-**A**-Q-L-K-Q-C
L2-12Q9ΔA	C-E-K-V-P-V-S-K-G-**A**-L-K-Q-C
L2-12L10ΔA	C-E-K-V-P-V-S-K-G-Q-**A**-K-Q-C
L2-12K11ΔA	C-E-K-V-P-V-S-K-G-Q-L-**A**-Q-C
L2-12Q12ΔA	C-E-K-V-P-V-S-K-G-Q-L-K-**A**-C

Amino acids are represented by their one letter code, reading left to right from amino to carboxyl termini. The analogue code, for example L2-12K9ΔA, refers to 12 residues from the native loop 2 sequence of BDNF with lysine at position 9 substituted with alanine. Cysteine residues not found in the native BDNF sequence were incorporated to form disulphide bridges, which are represented by lines between side chains.

[a]peptide L2-12P4ΔA not synthesized.

Example 4   Inhibition of BDNF-Mediated Sensory Neuron
             Survival by Monomeric Cyclic Loop 2 Peptides

The biological activity of the monomeric cyclic
loop 2 peptide analogues was assayed in primary cultures of
5   sensory neurons prepared from embryonic chick dorsal root
ganglia, essentially as described by Barde et al (1980).
The survival of specific subpopulations of these neurons in
culture is supported by neurotrophins acting through the
appropriate member of the trk receptor family (Lindsay,
10   1996). Briefly, 80 dorsal root ganglia were dissected from
four embryonic day 8-10 chicks (E8-E10), treated with 0.1%
trypsin for 20 min at 37°C, washed twice with 2 ml medium
(L-15 (CO_2), 5% horse serum, 60 µg/ml streptomycin and
100 µg/ml penicillin) and gently triturated. Non-neuronal
15   cells were removed by preplating the neuronal suspension on
a 10 cm tissue culture dish for 3 h at 37°C, 5% CO_2. Prior
to plating of neurons, 48-well tissue culture plates were
coated with poly-DL-ornithine (150 µl of 1 mg/ml in 0.15 M
sodium borate buffer pH 8.3) overnight at 4°C and then with
20   laminin (125 µl of 7.5 µg/ml in L-15 (CO_2)) for 4 h at
37°C, 5% CO_2. Immediately after removal of laminin
solution, 200 µl of suspension, and where appropriate,
samples of test compounds (2 µl) were added to each well.
After 1 h neurotrophic factors were added (either 2 or
25   6 µl), and viable neuron numbers were determined manually
by counting 40 standard fields at 200x magnification.

After 48 hrs incubation, phase-bright healthy
neurons with neurites at least twice the length of the cell
soma were counted in 20-30 fields at 200x magnification,
30   and counts expressed as a percentage of the original number
of viable neurons plated (% neuronal survival). Percentage
neuronal survival data was normalised, such that neuronal
survival in the presence of neurotrophin (4 x 10^{-11} M;
positive control) was set to 100%, while survival in the
35   absence of both neurotrophin and monomeric cyclic loop 2
peptide analogue (negative control) was set to 0%. Values
were expressed as mean ± SEM. Data from different

experiments were analysed for lack of significant variation using a parametric one-way analysis of variance (ANOVA) before being grouped. Statistics were performed using Instat version 2.04a (GraphPad, San Diego, CA, U.S.A.).

5   Prism software (GraphPad, San Diego, CA, U.S.A.) was used to fit sigmoidal curves to the data.

To investigate the ability of the loop 2 analogues L2-12, L2-12a, L2-10, L2-8 and L2-6 to modulate BDNF-mediated survival, the peptides were assayed from

10   1×10^{-11} to 1×10^{-4} M in competition with BDNF at 4×10^{-11} M, a concentration which produces near maximal survival. The results are summarised in Figure 3. All five peptides showed a similar pattern of concentration-dependent inhibition of BDNF-mediated survival, causing an

15   increase in inhibition from 1×10^{-11} to a maximum at approximately 1×10^{-6} M; above this concentration inhibition either reached a plateau (L2-10), or diminished (L2-12, L2-12a, L2-8 and L2-6), giving the concentration-response curve an inverted bell-shape. However, the

20   maximal level of inhibition produced by these peptides varied: L2-8 showed the greatest maximum (50% ± 5), followed by L2-12a (44% ± 4), L2-10 (41% ± 2), L2-12 (40% ± 3) and L2-6 (27% ± 6). The maximal inhibition and pIC_{50} values, the latter obtained from logistic sigmoidal

25   curves fit to the data, are summarised in Table 2.

Table 2

Summary of Data for L2-12, L2-12a, L2-10, L2-8
and L2-6 in Competition with BDNF in Cultures
of E8-E10 Sensory Neurons

5

Loop 2 analogue	Maximal inhibition	pIC_{50}
L2-12	40% ± 3	9.93 ± 0.15
L2-12a	44% ± 4	9.38 ± 0.30
L2-10	41% ± 2	8.16 ± 0.26
L2-8	50% ± 5	9.54 ± 0.16
L2-6	27% ± 6	10.53 ± 0.16

Maximal inhibition refers to the greatest % reduction in
normalised % BDNF-mediated neuronal survival. pIC_{50} values
were calculated from logistic sigmoidal curves fitted to
the data given in Figure 3.

In contrast to the results obtained with the monomeric cyclic peptides, the monomeric linear peptide L2-12b did not show significant inhibition of BDNF-mediated survival over the concentration range tested (1 x 10^{-11} to

5   1 x 10^{-4} M; Figure 3).

Example 5   Specificity of Inhibition of Neuronal Survival Activity by Monomeric Cyclic Loop 2 Analogues

10   To determine the specificity of the peptides in inhibiting BDNF-mediated survival, monomeric cyclic peptide L2-12 (1 x 10^{-11} to 1 x 10^{-4} M) was assayed in competition with NGF (4 x 10^{-11} M), using the assay described in Example 4. As shown in Figure 4, at the concentrations

15   tested peptide L2-12 did not significantly inhibit NGF-mediated survival. These data suggest that the inhibition of neuronal survival seen in Example 4 is specific for BDNF.

20   Example 6   Lack of Intrinsic Neuronal Survival Activity or Toxic Effects of Monomeric Cyclic Loop 2 Analogues

When added to cultures alone, ie. in the absence of neurotrophin, the monomeric cyclic peptide L2-12 neither

25   intrinsically promoted neuronal survival nor exhibited non-specific toxic effects on neurons at the concentrations tested (1 x 10^{-11} to 1 x 10^{-4} M), giving neuronal survival of around 5%, ie. similar to that of negative controls, as shown in Figure 4.

30   This lack of intrinsic neuronal survival promoting activity of the monomeric cyclic loop 2 peptide L2-12 was expected. Because L2-12 and the other monomeric cyclic loop 2 peptides are monomeric, they are unlikely to dimerise trk3, and the dimerization is crucial for trk-

35   mediated signalling (Jing et al, 1992).

Example 7   Effect of Monomeric Cyclic Peptides on the
              Concentration-Response Curve for BDNF

To examine the effect of inhibitory peptides on
the concentration-response curve of BDNF, monomeric cyclic

5   L2-12a (1×10^{-7} M) was added in competition with BDNF
(1.8×10^{-13} to 1.8×10^{-10} M in 0.5 log increments). The
results are shown in Figure 5. At this concentration
peptide L2-12a caused a significant depression ($\pm 0\%$) of the
maximal % neuronal survival response of BDNF from 41% to

10   24%, which is consistent with the maximal inhibition of
BDNF-mediated survival exhibited by L2-12a. The peptide
also caused a small rightward shift of the BDNF
concentration-response curve (pEC_{50} of BDNF alone:
11.2 ± 0.2; BDNF + L2-12a: 10.9 ± 0.3), although this was

15   not statistically significant.

Example 8   Identification of Amino Acids Important for
              the Inhibitory Effect of Monomeric Cyclic
              Loop 2 Peptides

20   The contribution of individual residues within
the monomeric cyclic L2-12 sequence towards BDNF-inhibitory
activity was examined by conducting an alanine scan (Ala
scan), and testing the resulting monomeric cyclic peptides
from 1×10^{-11} to 1×10^{-4} M in log increments for their

25   ability to modulate BDNF-mediated survival at 4×10^{-11} M.
The sequences of the alanine substituted peptides are shown
in Table 1 above, and the results are shown in Figure 6.

             A significant difference in maximal inhibition
compared to that produced by L2-12 (40% $\pm$ 3) was seen when

30   Ala was substituted for Val^3 (Val^{42} in native BDNF
sequence; 0% $\pm$ 9), $Ser^{6(45)}$ (2% $\pm$ 7), $Lys^{11(50)}$ (9% $\pm$ 4) and
$Gln^{12(51)}$ (5% $\pm$ 5), suggesting that these residues are
important for BDNF-inhibitory activity. Substitution of
Ala for $Val^{5(44)}$ yielded a peptide which gave a slight,

35   though insignificant, potentiation of BDNF-mediated
survival (-7% $\pm$ 9). However, the peptide did not show
intrinsic survival promoting activity in the absence of

BDNF (data not shown). No significant change in maximal inhibition was observed when Ala was substituted for $Glu^{1(40)}$ (39% ± 13), $Lys^{2(41)}$ (26% ± 7), $Lys^{7(46)}$ (33% ± 8), $Gly^{3(47)}$ (46% ± 13), $Gln^{9(48)}$ (32% ± 6) and $Leu^{10(49)}$ (33% ± 6).

Example 9   Molecular Design of Disulphide-linked Dimeric Bicyclic Loop 2 Analogues

The two loop 2 regions of BDNF are juxtaposed in the three-dimensional model of the dimer (Figure 1), which allows design of small dimeric peptides which mimic this spatial arrangement. On the basis of observations made in the highly analogous NGF-trkA receptor system, we predicted that small dimeric loop 2 analogues could act as agonists if they could facilitate dimerisation of trkB. It has been shown that divalent antibodies to trkA can cause the homodimerisation of this receptor, leading to signal transduction and NGF-like biological activity in vitro (Clary et al, 1994). Moreover, a small peptide mimetic of erythropoietin, produced by a recombinant library technique, possesses full erythropoietin-like biological activity as a result of self-association to form a dimer which dimerises the erythropoietin receptor (Wrighton et al, 1996). Examination of the X-ray crystal structure of the peptide-erythropoietin receptor complex (Livnah et al, 1996) reveals that the structure of the bound dimeric peptide bears a striking resemblance to the loop 2 regions of BDNF in our three-dimensional model.

The most effective of the monomeric, cyclic, disulphide-linked loop 2 peptides which were shown in Examples 4, 5, 7 and 8 were able to inhibit BDNF neuronal survival activity, peptide L2-8, was chosen as the basis for the design of dimeric peptides. This peptide consists of 8 amino acid residues of BDNF plus the two terminal cysteine residues oxidised to cyclic disulphide, ie. a total of 10 residues.

Examination of the model of the three-dimensional structure of BDNF revealed three amino acid positions in which the two loop 2 regions in BDNF are in close proximity, thus presenting an opportunity to create dimeric

5   analogues of peptide L2-8. The positions, corresponding to Pro^2, Val^3 and Ser^4 of peptide L2-8, are characterised by $C\alpha$-to-$C\alpha$ distances of 11.3Å, 5.4 Å and 6.2 Å, respectively, as shown in Figure 7A. Conformational analysis of a cystine residue (*ie.* two disulphide-linked

10   cysteine residues) by computational chemical methods revealed that the mean $C\alpha$-to-$C\alpha$ distance of this residue was 5.4Å (90% CI: 5.22-5.44Å), as shown in Figure 7B. These data suggested to us that a cystine residue could comfortably be incorporated into peptide L2-8 at positions

15   Val^3 and Ser^4, to give dimeric, disulphide-inked peptides that might mimic the spatial arrangement of the loop 2 regions in native BDNF, but fitted considerably less well in place of Pro^2.

However, as shown in Example 8, examination of a

20   series of peptides based on a 14 amino acid monomeric loop 2 peptide inhibitor of BDNF action, in which amino acids were systematically replaced with Ala, showed that residues equivalent to Val^3 and Ser^4 in peptide L2-8 were required for BDNF inhibitory activity, and therefore were presumably

25   involved in binding to BDNF receptors. No data were available concerning an Ala replacement at a position equivalent to Pro^2.

On the basis of the structural data alone, we chose to synthesise the, disulphide-linked dimeric bicyclic

30   peptides $(L2-8V3C)_2$ and $(L2-8S4C)_2$, incorporating a cystine bridge in place of Val^3 and Ser^4 respectively. The disulphide-linked dimeric bicyclic peptide $(L2-8P2C)_2$, in which the cystine linkage was incorporated in place of Pro^2, was also synthesised, as definitive information on

35   the role of Pro^2 was not available, even though our structural data suggested that cystine would not provide the optimal means of dimerisation at this point.

Example 10   Synthesis of Disulphide-Linked Dimeric
   Bicyclic Loop 2 Analogues

The disulphide-linked dimeric bicyclic peptides
(L2-8P2C)$_2$, (L2-8V3C)$_2$ and (L2-8S4C)$_2$ were synthesised by
standard solid phase synthesis techniques using Fmoc amino
acids, as described in Example 3, and using a mixed Cys
protection strategy (Cys(Trt) and Cys(Acm)). The general
method is illustrated in Scheme 1.

5

10

Scheme 1

Synthesis of disulphide-linked dimeric bicyclic peptide
analogues of loop 2 of BDNF

5

H-Cys(Trt)————Cys(Acm)————Cys(Trt)——Resin

⬇ 90% TFA + scavengers

H-Cys————Cys(Acm)————Cys-OH

⬇ 10% DMSO pH 8.4

H-Cys————Cys(Acm)———— Cys-OH

⬇ I$_2$ 50mM in Acetic acid
quench

H-Cys———— Cys————Cys-OH

H-Cys————Cys————Cys-OH
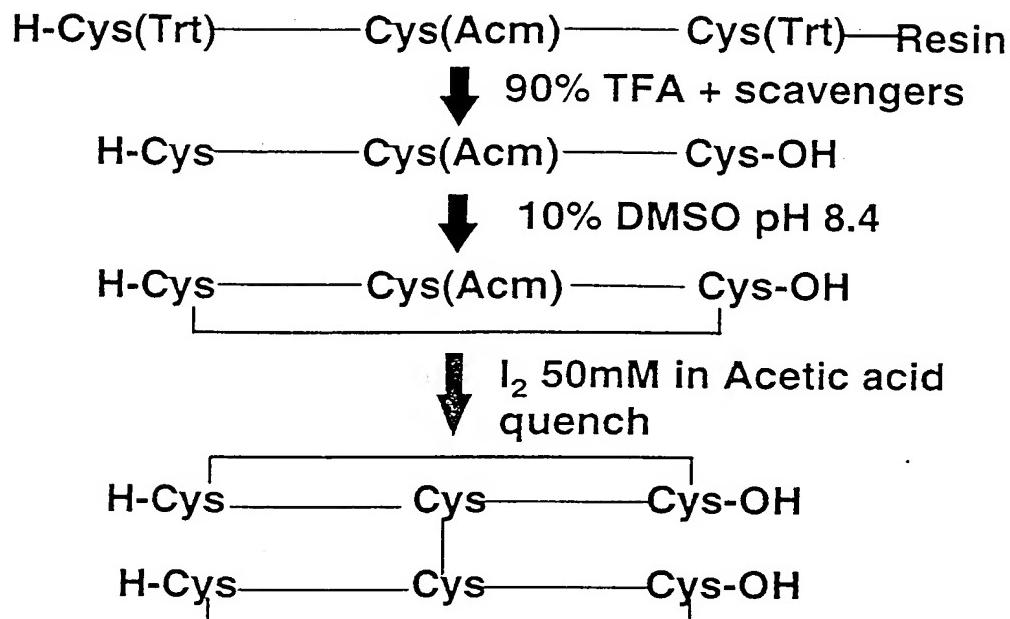
Peptides and intermediates were purified by reverse-phase high performance liquid chromatography, and characterised by electrospray mass spectrometry. The structures of the compounds synthesised are shown in Table 3.

Table 3   Structures of Disulphide-linked Dimeric Bicyclic
Loop 2 Analogues and Their Monomeric Cyclic Precursors

5

10

L2-8

(L2-8P2C)₂

(L2-8V3C)₂

(L2-8S4C)₂

L28P2C(Acm)

L28S4C(Acm)

L2-8   C-V-P-V-S-K-G-Q-L-C

(L2-8P2C)₂   C-V-C-V-S-K-G-Q-L-C   C-V-C-V-S-K-G-Q-L-C

(L2-8V3C)₂   C-V-P-C-S-K-G-Q-L-C   C-V-P-C-S-K-G-Q-L-C

(L2-8S4C)₂   C-V-P-V-C-K-G-Q-L-C   C-V-P-V-C-K-G-Q-L-C

L28P2C(Acm)   C-V-C(Acm)-V-S-K-G-Q-L-C

L28S4C(Acm)   C-V-P-V-C(Acm)-K-G-Q-L-C
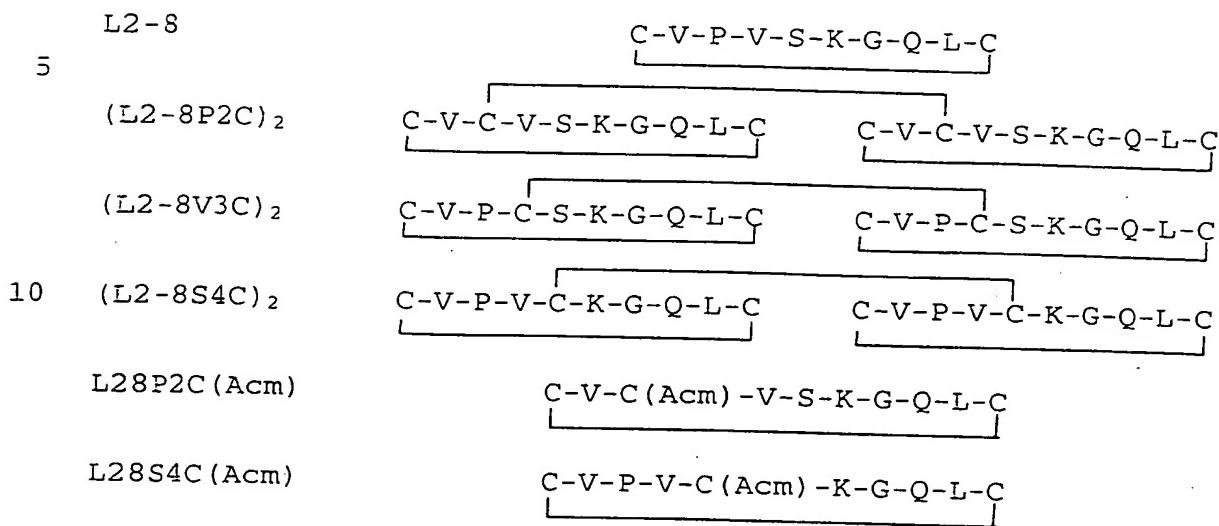
Example 11   Intrinsic Neuronal Survival Activity of
              Disulphide-Linked Dimeric Bicyclic Loop 2
              Analogues

The disulphide-linked dimeric bicyclic loop 2
peptides were analysed for their ability to promote the
survival of sensory neurons in cultures prepared from
dorsal root ganglia obtained from embryonic day 8-10
chicks, as described in Example 4. Peptides (L2-8P2C)$_2$ and
(L2-8S4C)$_2$ each displayed concentration-dependent neuronal
survival activity, maximally promoting the survival of 28%
and 30% of the neurons that would be supported by BDNF
itself. These results are shown in Figure 8.

The activity of these two disulphide-linked
dimeric bicyclic loop 2 peptides was surprising in view of
the number of the modifications. In peptide (L2-8S4C)$_2$, the
serine residue (Ser^4) shown to be important for the
inhibitory action on BDNF-mediated neuronal survival of the
monomeric cyclic loop 2 peptides (see Example 8) was
replaced by the disulphide-linked cystine residue. In
peptide (L2-8P2C)$_2$, the Cα-to-Cα distance of the cystine
residue is likely to be much shorter than the corresponding
distance in our model of BDNF of the two proline residues
which it replaces.

In contrast, peptide (L2-8V3C)$_2$ was inactive, as
shown in Figure 8, despite the likelihood that it could
best accommodate the cystine residue, at least in terms of
interatomic distance.

Example 12   Lack of Intrinsic Neuronal Survival Activity
              of Monomeric Precursors of Dimeric Bicyclic
              Loop 2 Analogues

To determine whether the dimeric nature of the
disulphide-linked dimeric bicyclic loop 2 peptides was
required for intrinsic neuronal survival activity, we
assayed peptides L2-8P2C(Acm) and L2-8S4C(Acm), the
monomeric cyclic precursors of peptides (L2-8P2C)$_2$ and (L2-
8S4C)$_2$ in which the Acm groups on the internal Cys were

intact, for their ability to promote sensory neuronal
survival in culture. Unlike their dimeric counterparts,
both monomeric cyclic peptides were inactive, as shown in
Figure 9.

5

Example 13   Molecular Design of an Amide-Linked Dimeric
                 Bicyclic Loop 2 Analogue
          Because of the position of the cysteine residue
in the disulphide-linked dimeric bicyclic loop 2 analogues
10   described in Examples 9-11 in the region required for the
inhibitory activity of the monomeric cyclic peptides, we
further examined the BDNF model to see if other dimeric
bicyclic peptides could be designed that did not involve
the replacement of these possible receptor-binding
15   residues. On the basis of $C\alpha$-to-$C\alpha$ distance measurements,
we reasoned that an amide-linked dimeric bicyclic peptide,
$(L2-8\&E+K)_2$, could be created by joining together two
analogues of the monomeric cyclic peptide L2-8 via an amide
bond between an additional lysine and glutamate residue
20   added to the C-terminus. An illustration of this is shown
in Table 4.

Table 4   <u>Structure of disulphide-linked bicyclic</u>
                 <u>loop 2 analogue</u>

5   (L2-8&E+K)$_2$

Ac-C-V-P-V-S-K-G-Q-L-C-E-NH$_2$ Ac-C-V-P-V-S-K-G-Q-L-C-K-NH$_2$
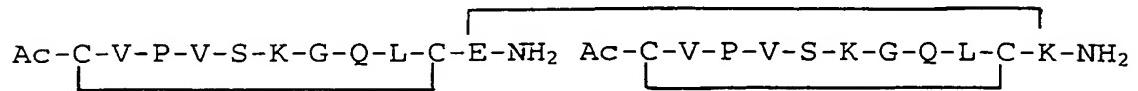
Example 14   Synthesis of an Amide-Linked Dimeric
              Bicyclic Loop 2 Analogue

The amide-linked dimeric bicyclic peptide $(L2-8\&E+K)_2$ was prepared as shown in Scheme 2 by condensing two
5   cyclic N-acetylated, C-amidated, partially-protected
monomers synthesised by standard solid phase techniques on
Rink amide MBHA resin as described in Example 3.

Scheme 2

Synthesis of an amide-linked dimeric bicyclic loop 2
5   analogue

assembly   *Fmoc solid phase assembly*

Ac-C(Trt)------S(Bzl)-----K(Z)----C(Trt)-K(Boc)-(R)   Ac-C(Trt)----S(Bzl)-----K(Z)----C(Trt)-E(Trt)-(R)

cleavage   *TFA + scavengers*

Ac-C-------S(Bzl)------K(Z)-----C--K-NH₂

**oxidation
(cyclisation)**   *DMSO in NH₄CO₃ solution*

Ac-C--------S(Bzl)---------K(Z)----C--K-NH₂   Ac-C--------S(Bzl)-------K(Z--)----C--E-NH₂

**dimerisation
reaction**   *HATU & DIEA in DMF*

Ac-C-------S(Bzl)--------K(Z)------C--K-NH₂   Ac-C-------S(Bzl)-------K(Z)------C--E-NH₂

cleavage   *HF + m-cresol*

Ac-C-------S-----K----------C--K-NH₂   Ac-C-------S----K----------C--E-NH₂
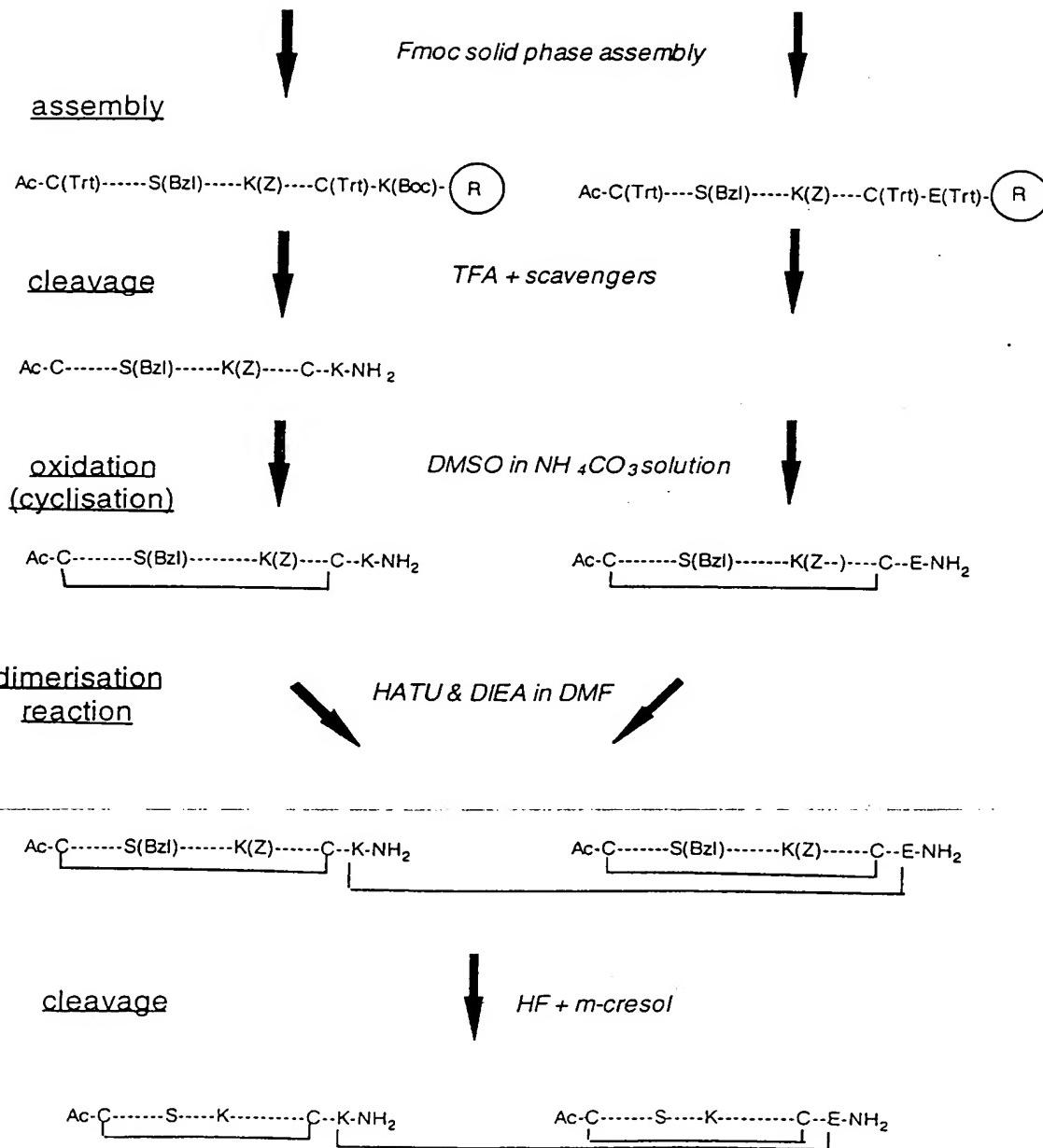
The remaining Ser and Lys protecting groups were removed by treatment of the partially protected bicyclic dimer with hydrogen fluoride/m-cresol (10:1) for one hour at 5°C. HF was removed by evaporation at room temperature. The
5 desired peptide (L2-8&E+K)$_2$ and intermediates were purified by HPLC and characterised by mass spectrometry.

Example 15   Intrinsic Neuronal Survival Activity
                    of an Amide-Linked Dimeric Bicyclic Loop
10   2 Analogue

        The amide-linked dimeric bicyclic loop 2 peptide (L2-8&E+K)$_2$ was assayed in cultures of sensory neurons prepared from dorsal root ganglia obtained from embryonic chicks, as described in Example 4. Peptide displayed
15 concentration dependent neuronal survival activity, supporting the survival of 28% of those neurons supported by BDNF (1ng/ml) with an EC$_{50}$ in the order of 10^{-8} M. The results are shown in Figure 10.

        This activity was similar both in maximal effect
20 and potency to that observed with the disulphide-linked dimeric bicyclic loop 2 analogues (L2-8P2C)$_2$ and (L2-8S4C)$_2$ described in Example 11. Thus it appears that different chemical linkers incorporated in different positions within the dimeric bicyclic loop 2 analogues imparts neuronal
25 survival promoting activity to these peptides.

Example 16   Molecular Design of a Dimeric Tricyclic
                    Loop 2 Analogue

        Although the dimeric bicyclic peptides described
30 in Examples 9 through 11 and 13 through 15 represent a significant step in the discovery of small molecules which mimic the action of BDNF, they are nonetheless considerably less potent efficacious than the parent peptide. One reason for the reduced activity of the dimeric bicyclic
35 peptides compared to BDNF could be their ability to rotate relatively freely about their dimerising constraint, be it a Cys-to-Cys disulphide or a Lys-to-Glu amide. To try and

create a molecule that might show either improved efficacy (as evidenced by an increase in the maximal percent neuronal survival) or increased potency, we reasoned that we would need to restrict the freedom of rotation about the

5   dimerising constraint. To do this, we chose to combine, in one molecule, the two different dimerising constraints used in the disulphide-linked and the amide-linked dimeric bicyclic loop 2 analogues. We anticipated that the resultant dimeric tricyclic loop 2 analogue (L2-8S4C&E+K)$_2$,

10   by restricting the rotation of the two loop 2 moieties relative to one another would much better mimic the loop 2 orientation seen in the native protein, and therefore would show improved efficacy and potency. This is shown in Table 5.

15

Table 5 Structure of Dimeric Tricyclic Loop 2 Analogue

(L2-8S4C&E+K)$_2$

5   Ac-C-V-P-V-C-K-G-Q-L-C-E-NH$_2$ Ac-C-V-P-V-C-K-G-Q-L-C-K-NH$_2$
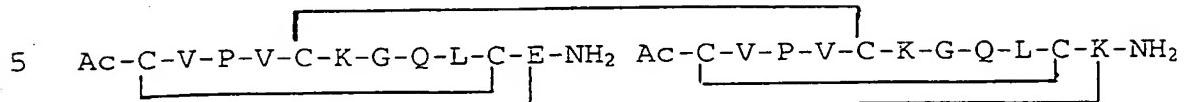
Example 17   Synthesis of a Dimeric Tricyclic Loop 2
              Analogue

The dimeric tricyclic peptide (L2-8S4C&E+K)$_2$ was
prepared as shown in Scheme 3 from two cyclic N-acetylated,
5   C-amidated, partially-protected monomers synthesised by
standard solid phase techniques on Rink amide MBHA resin
as described in Example 14.

Scheme 3

Synthesis of a dimeric tricyclic loop 2 analogue

assembly   ⬇   *Fmoc solid phase assembly*   ⬇

Ac-C(Trt)-------C(Acm)------K(Z)---C(Trt)· Ⓡ Ac-C(Trt)——C(Acm)------K(Z)----C(Trt)- Ⓡ

cleavage   ⬇   *TFA + scavengers*   ⬇

Ac-C-------C(Acm)------K(Z)-------C--K- $_2$ Ac-C--------C(Acm)------K(Z)-------C--E- $_2$

oxidation
(cyclisation)   ⬇   *DMSO in NH$_4$CO$_3$ solution*   ⬇

Ac-C-------C(Acm)------K(Z)-----C--K- $_2$ Ac-C-----C(Acm)------K(Z)-----C--E- $_2$

HATU & DIEA in DMF

Ac-C-------C(Acm)-- ---K(Z)----C--K- $_2$ Ac-C-------C(Acm)------K(Z)----C--E- $_2$

2nd dimerisation
reaction   ⬇   *i I$_2$ + AcOH*
ii ascorbic acid

Ac-C-------C------K(Z)----C--K- $_2$ Ac-C------C-----K(Z)----C--E- $_2$

cleavage   ⬇   *HF + m-cresol*

Ac-C-------C-----K---------C--K- $_2$ Ac-C-------C-----K---------C--E- $_2$
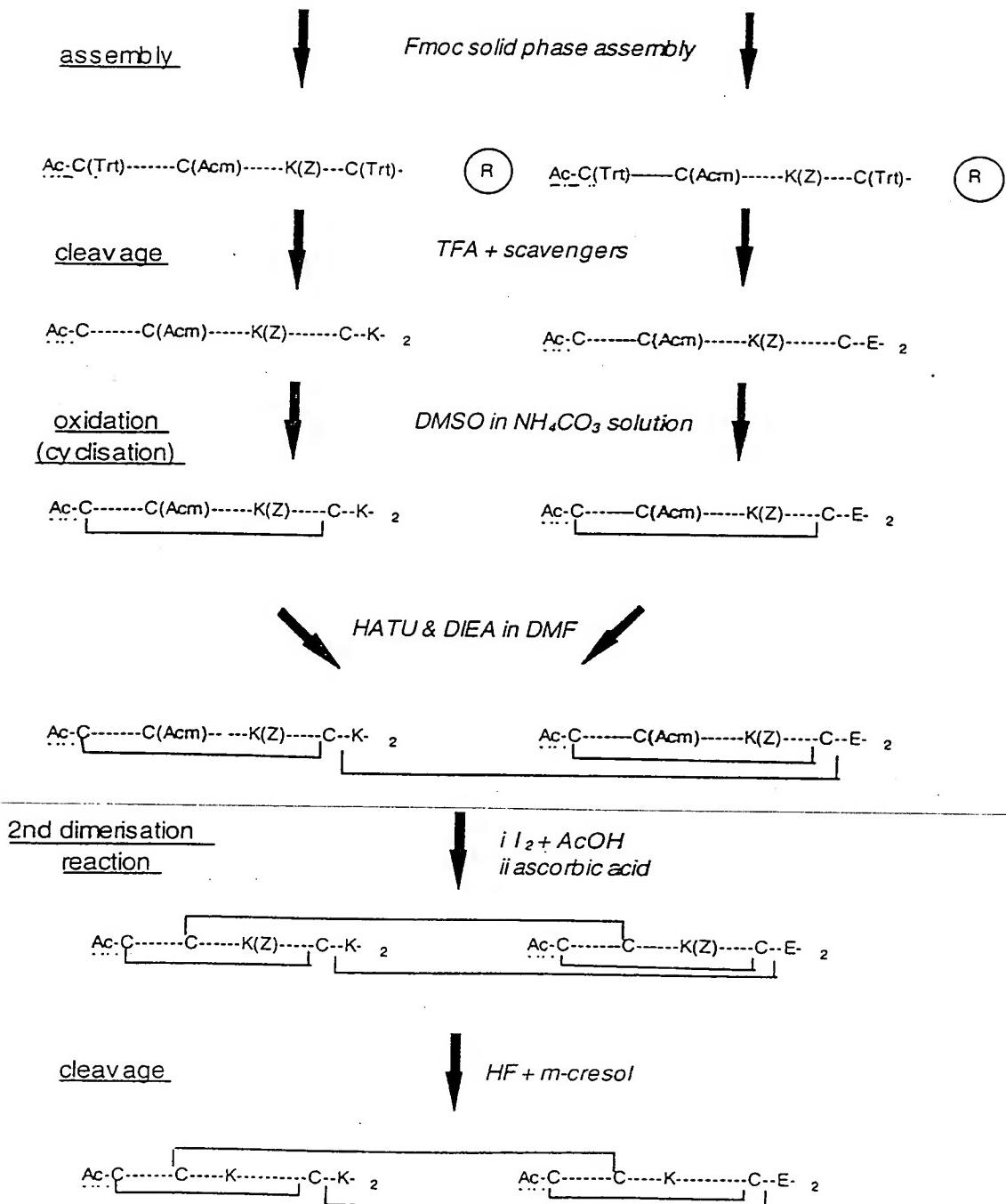
The monomers were initially condensed in the first dimerisation reaction via the free lysine and glutamate sidechains. A second dimerisation reaction was carried by oxidising the internal cysteine residues, completing the tricycle. The remaining Lys protecting groups were removed by treating the partially protected tricyclic dimer with hydrogen fluoride/m-cresol (10:1) for one hour at 5°C. HF was removed by evaporation at room temperature. The desired peptide (L2-8S4C&E+K)$_2$ and intermediates were purified by HPLC and characterised by mass spectrometry.

Example 18   Intrinsic Neuronal Survival Activity of a Dimeric Tricyclic Loop 2 Analogue

The dimeric tricyclic loop 2 peptide (L2-8S4C&E+K)$_2$ was assayed in cultures of sensory neurons prepared from dorsal root ganglia obtained from embryonic chicks, as described in Example 4. Peptide displayed concentration dependent neuronal survival activity, supporting the survival of 35% of those neurons supported by BDNF (1ng/ml) with an EC$_{50}$ in the order of 10^{-10} M. The results are shown in Figure 11.

The maximal neuronal survival promoting effect of the dimeric tricyclic loop 2 peptide (L2-8S4C&E+K)$_2$ is similar to that of of the dimeric bicyclic loop 2 analogues. However peptide (L2-8S4C&E+K)$_2$ is approximately two orders of magnitude more potent than the dimeric bicyclic analogues. This activity is consistent with the hypothesis that the presence of two dimerising constraints (Cys-to-Cys disulphide and Lys-to-Glu amide) would create a molecule which much better mimics the spatial arrangement of the two loop 2 moieties than any of the dimeric bicyclic compounds, which contain only a single dimerising constraint.

Example 19   Molecular Design of Monomeric Cyclic
              Analogues of the p75 Binding Region of Loop 4
              of BDNF

The three positively-charged residues thought to
be important for the binding of BDNF to the low affinity
neurotrophin receptor p75 are contiguous (Lys^{95}-Lys^{96}-Arg^{97}),
and are located on loop 4, as shown in Figure 1. This gave
us the opportunity to propose small monomeric cyclic
peptides that might mimic the conformation of this
tripeptide sequence, using the computer-aided molecular
design approach described in Example 2. On the basis of
these studies we chose to synthesise two cyclic monomeric
peptides: L4-3pA, a pentapeptide incorporating a relatively
conformationally restricted DPro residue; and L4-3Hx, a
tetrapeptide incorporating a conformationally flexible 6-
aminohexanoyl residue. Both peptides were cyclised by
condensing their amino-terminus with their carboxy-terminus
(head-to-tail cyclisation).

Example 20   Synthesis of Monomeric Cyclic Analogues of
              the p75 Binding Region of Loop 4 of BDNF

The monomeric cyclic loop 4 peptides were
synthesised from 9-fluorenylmethoxycarbonyl (Fmoc) amino
acids, using standard solid phase synthesis protocols as
described in Example 3. The linear sidechain-protected
peptides L4-3pAa and L4-3Hxa, suitable for head-to-tail
cyclisation to give the monomeric cyclic peptides L4-3pA
and L4-3Hx respectively, were obtained by treating peptides
synthesised on acid labile 2-chlorotrityl derivatised resin
(NovaBiochem, Australia) with acetic
acid/trifluoroethanol/dichloromethane (1:1:8) for 30
minutes (Barlos et al., 1991). The cyclic peptides were
obtained by stirring the appropriate linear side chain-
protected peptide (0.1 to 0.5 mg/ml) in dichloromethane in
the presence of the standard peptide bond-formation
reagents 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyl
uronium hexafluorphosphate (HBTU), 1-hydroxybenzotriazole

(HOBt) and diisopropylamine (DIEA) (HBTU:HOBt:DIEA 1:1:1.5 equivalents relative to peptide). Treatment of the product of this reaction with TFA/scavengers yielded the desired fully-deprotected product. The corresponding linear

5 homologues were prepared by treating the appropriate sidechain-protected linear peptide with TFA/scavengers, without prior cyclisation.

Cyclisation reactions were monitored and peptides purified by reverse phase HPLC on either analytical (4.6 mm

10 internal diameter) or semi-preparative (22.5 mm) C18 columns, using linear acetonitrile gradients in 0.1% TFA solution at appropriate flow rates. Desired fractions were collected and lyophilised for characterisation by mass spectrometry.

15 Synthesis of peptide L4-3pA yielded two stereoisomers, L4-3pA(I) and L4-3pA(II), each with the desired molecular weight of 581 daltons. These isomers were purified by HPLC and were assayed seperately for biological activity.

20 A list of the compounds synthesised is given in Table 6.

Table 6   Structures of Monomeric Cyclic Loop 4
           Analogues and Their Linear Homologues

L4-3pA(I)

and   ⌐DPro-Ala-Lys-Lys-Arg⌐
                            └_____┘
L4-3pA(II)

L4-3Hx   ⌐Ahx-Lys-Lys-Arg⌐
                                └_____┘

L4-3pAa   H- DPro-Ala-Lys-Lys-Arg-OH

L4-3Hxa   H-Ahx-Lys-Lys-Arg-OH

All amino acid residues are given by their standard three
letter codes, except Ahx: 6-amino hexanoyl.

Example 21   Intrinsic Neuronal Survival Activity of a
              Monomeric Cyclic Analogue of the p75 Binding
              Region of Loop 4 of BDNF

The monomeric cyclic analogues of the p75 binding
region of loop 4, L4-3pA(I), L4-3pA(II), L4-3Hx and their
linear homologues L4-3pAa and L4-3Hxa, were assayed in
cultures of sensory neurons prepared from embryonic chicks
as described in Example 4. As shown in Figure 10, the
monomeric cyclic loop 4 peptide, L4-3pA(II) displayed
concentration-dependent neuronal survival activity. This
intrinsic neuronal survival activity of L4-3pA(II) was
surprising; unlike the loop 2 peptides decribed in Example
11, it is neither dimeric nor bicyclic. Moreover, the
activity was confined to L4-3pA(II). Neither its
stereoisomer L4-3pA(I), the other monomeric cyclic loop 4
peptide L4-3Hx constrained by the more conformationally
flexible aminohexanoyl residue, nor their linear
counterparts displayed neuronal survival activity in this
assay system, as shown in Figure 11.

Example 22   Lack of Inhibition of BDNF-Mediated Sensory
              Neuron Survival by Monomeric Cyclic Loop 4
              Analogues of the p75 Binding Region of Loop 4
              of BDNF and Their Linear Homologues

The monomeric cyclic analogues of the p75 binding
region of loop 4 L4-3pA(I), L4-3pA(II), L4-3Hx and their
linear homologues L4-3pAa and L4-3Hxa, were assayed for
their ability to modulate the neuronal survival effects of
BDNF and NGF in cultures of sensory neurons prepared from
embryonic chicks as described in Example 4. Unlike the
monomeric cyclic loop 2 peptides, none of the monomeric
cyclic loop 4 peptides or their linear homologues showed
any significant inhibition of either BDNF- or NGF- mediated
neuronal survival, as shown in Figures 12 and 13,
respectively.

Example 23   Role of DPro in the Neuronal Survival
              Promoting Activity of Monomeric Cyclic Loop
              4 Analogues of the p75 Binding Region of
              BDNF

5   Given the biological data obtained with L4-
3pA(II) described in Example 21, we decided to investigate
the role of the DPro residue in the neuronal survival
activity of the monomeric cyclic loop 4 peptides. We chose
to synthesise two compounds using the methods described in
10   Example 20: L4-3Ap, in which the position of the DPro
residue is swapped with the Ala residue; and L4-3AP, in
which the configuration of the Pro residue is L rather than
D.   In a manner presumably analogous to that seen with L4-
3pA, both peptides yielded two isomers if identical
15   molecular weight: L4-3Ap(I), L4-3Ap(II), L4-3AP(I) and L4-
3AP(II). The sequences of these four peptides is shown in
Table 7.

Table 7 Structure of Further Monomeric Cyclic Loop 4
Analogues

L4-3Ap(I)
5   and   ⌐Ala-DPro-Lys-Lys-Arg⌐
     L4-3Ap(II)   └_____┘

     L4-3AP(I)
     and   ⌐Ala-Pro-Lys-Lys-Arg⌐
10   L4-3AP(II)   └_____┘

The monomeric cyclic peptides were assayed in cultures of sensory neurons prepared from embryonic chicks as described in Example 4. Unlike peptide L4-3pA(II), neither L4-3Ap(I), L4-3Ap(II), L4-3AP(I) nor L4-3AP(II)

5 displayed neuronal survival activity. These data, shown in Figure 16, suggest that both the position in the cyclic sequence and stereochemistry of the Dpro residue are important for the neuronal survival activity displayed by peptide L4-3pA(II).

10

Example 24   Role of Lys Residues in the Neuronal
                Survival Promoting Activity of Monomeric
                Cyclic Loop 4 Analogues of the p75 Binding
                Region of BDNF

15   To investigate the importance of the two Lys residues to the neuronal survival activity of the monomeric cyclic loop 4 peptides, we chose to synthesise two analogues of peptide L4-3pA using the methods described in Example 20 in which a Lys residue is replaced by Ala: L4-

20 3K3ApA and L4-3K4ApA. Unlike L4-3pA, both L4-3K3ApA and L4-3K4ApA yielded only single major products following synthesis and cyclisation. The sequences of these two peptides is shown in Table 8.

25

Table 8   Structure of Further Monomeric Cyclic Loop 4
Analogues Incorporating Ala for Lys

L4-3K3pA   ⌐DPro-Ala-Ala-Lys-Arg⌐
                        └_____┘

5

L4-3K4ApA   ⌐-DPro-Ala-Lys-Ala-Arg⌐
                        └_____┘

The monomeric cyclic peptides L4-3K3pA and L4-3K4ApA were assayed in cultures of sensory neurons prepared from embryonic chicks as described in Example 4. Compared to L4-3pA(II), peptides L4-3K3pA and L4-3K4ApA displayed

5 only marginal neuronal survival activity. These data, shown in Figure 17, suggest that the two Lys residues of the cyclic monomeric peptide L4-3pA are required for neuronal survival activity.

10 Example 25   NMR Analysis of Monomeric Cyclic Analogue
of the p75 Binding Region of Loop 4 of BDNF
The neuronal survival activity of the monomeric cyclic analogues of the p75 binding region of loop 4 of BDNF is confined almost exclusively to peptide L4-3pA(II).

15 To examine a structural basis for this neuronal survival activity, we chose to determine the structure of peptide L4-3pA(II) in solution using NMR techniques. An HPLC pure samples of peptide L4-3pA(II) was lyophilised then taken up in 550 ml of $10\%^2H_2O/90\%H_2O$ and the pH adjusted to 5.3. The

20 solution was then transferred into a 5mm NMR tube. NMR spectra were acquired at 400 MHz on a Varian Inova 400 MHz NMR spectrometers. One-dimensional ^1H spectra were acquired with a sweep-width of 4000Hz over 8K points. Solvent suppression was achieved with selective low-power

25 presaturation. Spectra were acquired at a series of sample temperatures (30º C, 15º C and 5º C) to check for temperature dependence of the peptide spectrum. The peptide did not show significant temperature dependence. All subsequent spectra were recorded at 30º C.

30 A series of 2D ^1H spectra were then recordedfor L4-3pA. Typically, each spectrum was acquired with a sweep width of 4000 Hz over 1024 points, with 800 t_1 increments. TOCSY and DQF-COSY spectra were acquired for use in spin system assignments while ROESY spectra were acquired to

35 generate distance constraints. Spectra were initially transformed using the Varian VNMR software package to check for the quality of the data. Subsequently spectra were

transformed using NMRpipe and analysed using NMRview. Complete assignment of all non-exchangeable proton resonances was made. Dihedral constraints for the backbone Φ angles were derived from the J^3_{NH-CaH} coupling constants

5 measured from 1D spectra. A total of 61 structurally important distance constraints and 3 backbone Φ angle constraints were determined from the NMR data for L4-3pA(II).

Structure calculation was carried out using the
10 software package DYANA. Cyclisation of the peptide was achieved by introducing a set of special distance constraints to both bring the ends of the peptide together and restrain the peptide bond angle to 180º. A modified version of the residue library containing a set of
15 parameters defining a DPro residue was produced to allow calculation to include the DPro residue. A total of 100 structures were calculated on the basis of the NMR-derived constraint list by 10000 steps of simulated annealing followed by 2000 steps of minimisation of the DYANA target
20 function. The 20 structures with the lowest target function were then selected as the final family of structures for the peptide. An overlay of these structures of peptide L4-3pA(II) can be found in Figure 18.

As can be seen in Figure 18, the conformation of
25 the backbone of peptide peptide L4-3pA(II) is uniquely defined in solution. In addition, sidechain of Lys[4] is adopts a single conformation up to its gamma-carbon atom, while the conformation of the sidechain of Arg[5] is uniquely defined to the delta-nitrogen. The presence of a single
30 backbone conformation and well-defined sidechains for peptide L4-3pA is consistent with the biological data showing that compounds of closely related sequence to L4-3pA show either markedly reduced or no neuronal survival activity in cell culture experiments. This exceptionally
35 well-defined conformation of L4-3pA will be used as a template for the design of non-peptidic molecules with neuronal survival promoting activity.

Example 26   *In vivo efficacy of Peptides*

The ability of the dimeric bicyclic analogues of loop 2 of BDNF, dimeric tricyclic analogues of loop 2 of BDNF and monomeric cyclic analogues of the p75 binding
5   region of loop 4 of BDNF to prevent or slow neurodegeneration are tested in suitable *in vivo* assays, such as that of Cheema *et al.* (1994a and 1994b). In a typical experiment, newborn (24-48h) Wistar rat pups are rendered unconscious by ice-induced hypothermia. The
10   median and ulnar nerve in the right forelimb are exposed, transected and wrapped with a piece of gel foam containing the peptide analogue to be tested at a dose determined from the *in vitro* studies. Control animals receive gel foam containing either saline or an equivalent dose of an
15   inactive peptide. Pups are re-united with their mothers and after 3 or 5 days are killed with a lethal injection of sodium pentobarbital (150 mg/kg) and perfused with a buffered 4% solution of paraformaldehyde. DRGs from the eighth cervical and fifth lumbar levels are dissected out
20   and embedded in paraffin. Serial transverse sections are cut, mounted on glass slides and stained with 0.5% cresyl violet. Neurons displaying prominent nucleoli are counted in every fifth section to include the entire rostrocaudal length of the DRG. Effects of peptides on neuronal loss
25   are determined by comparing the number of neurons in the experimental side versus that in the intact contralateral DRGs. Each group contains eight animals, and up to five compounds (with one control group of eight) can be tested at any one time. Student's t-test is then used to
30   determine statistical significance.

It will be apparent to the person skilled in the art that while the invention has been described in some detail for the purposes of clarity and understanding,
35   various modifications and alterations to the embodiments and methods described herein may be made without departing from the scope of the inventive concept disclosed in this

specification.

       References cited herein are listed on the
following pages, and are incorporated herein by this
5   reference.

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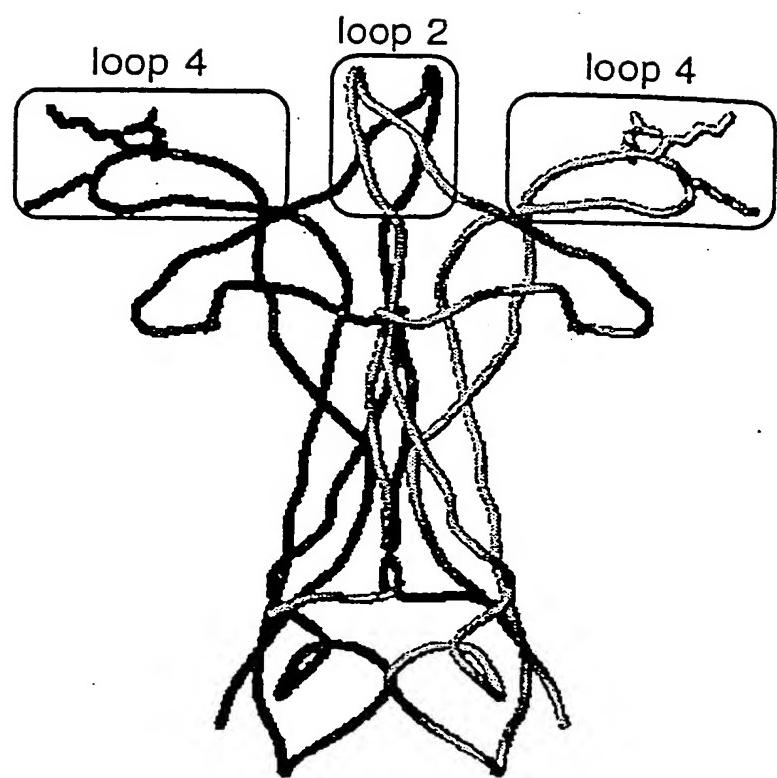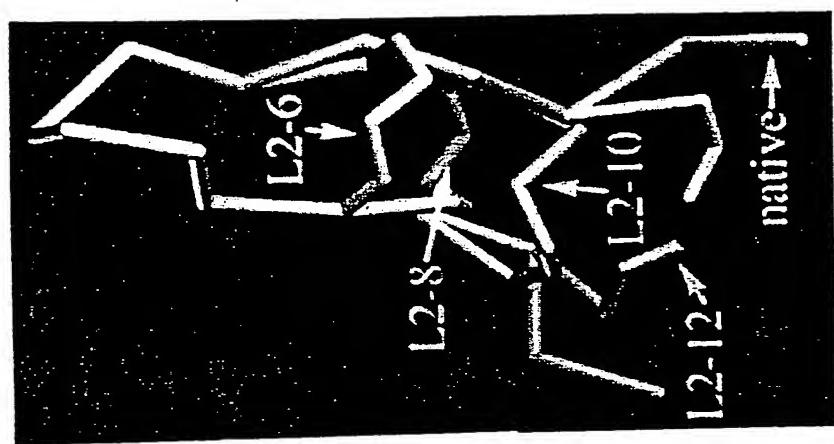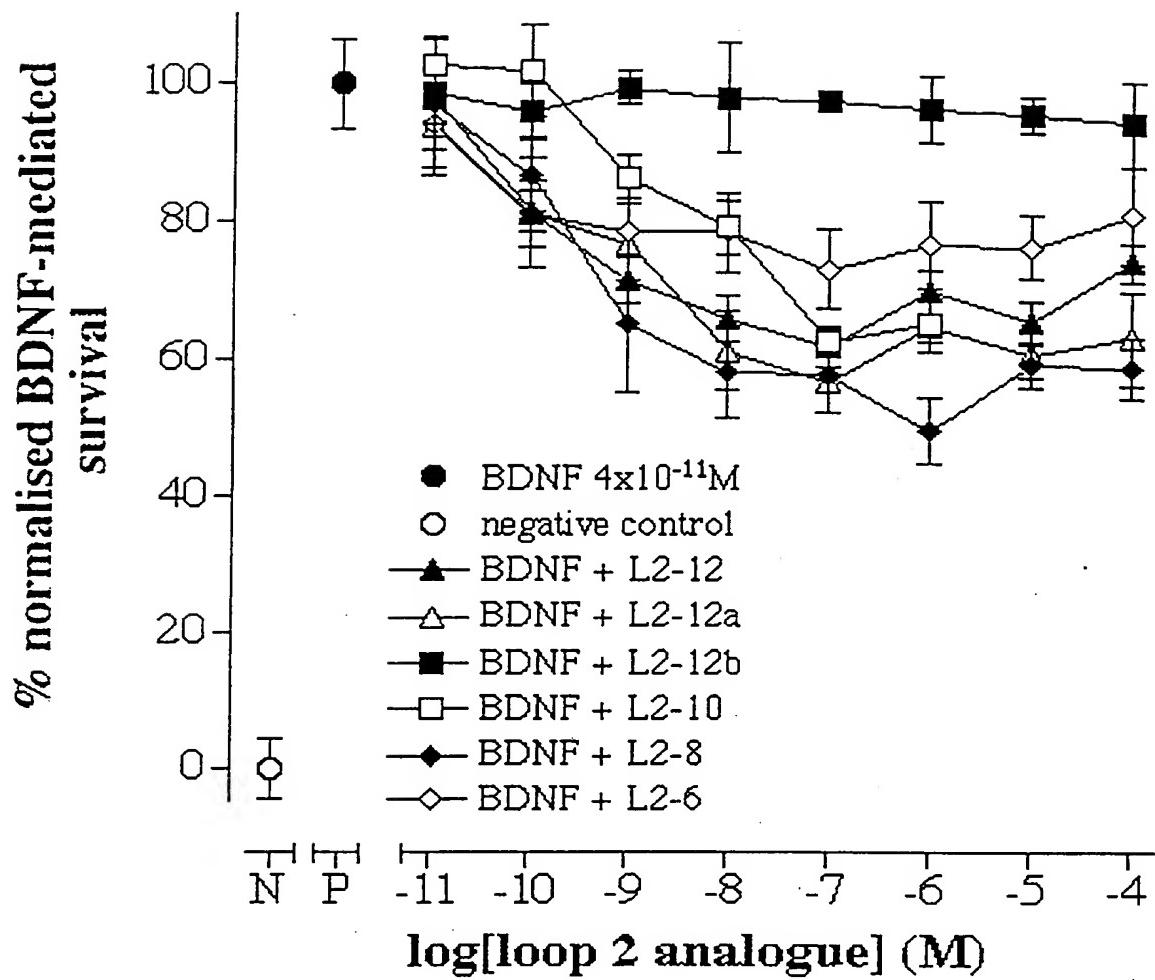
FIGURE 1

FIGURE 2

FIGURE 3
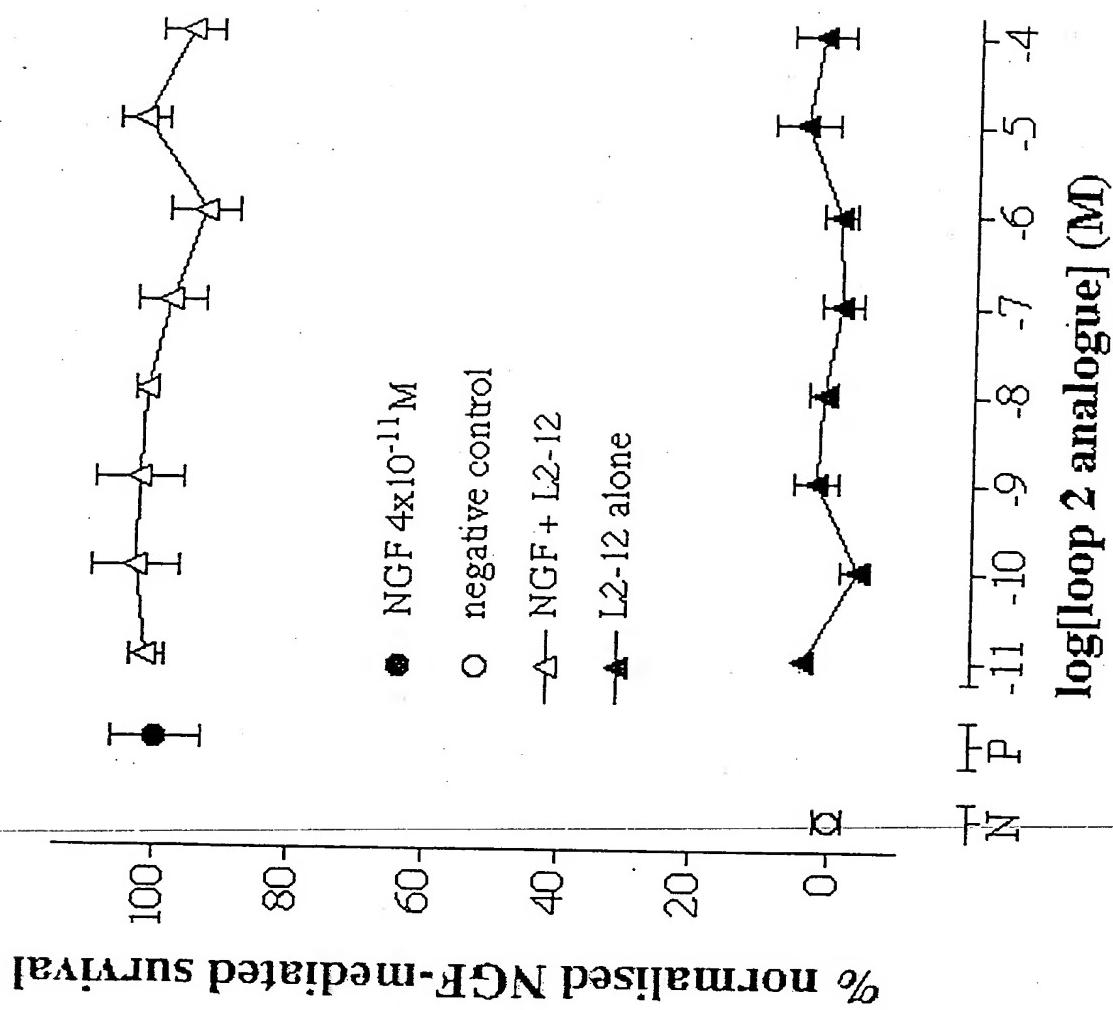
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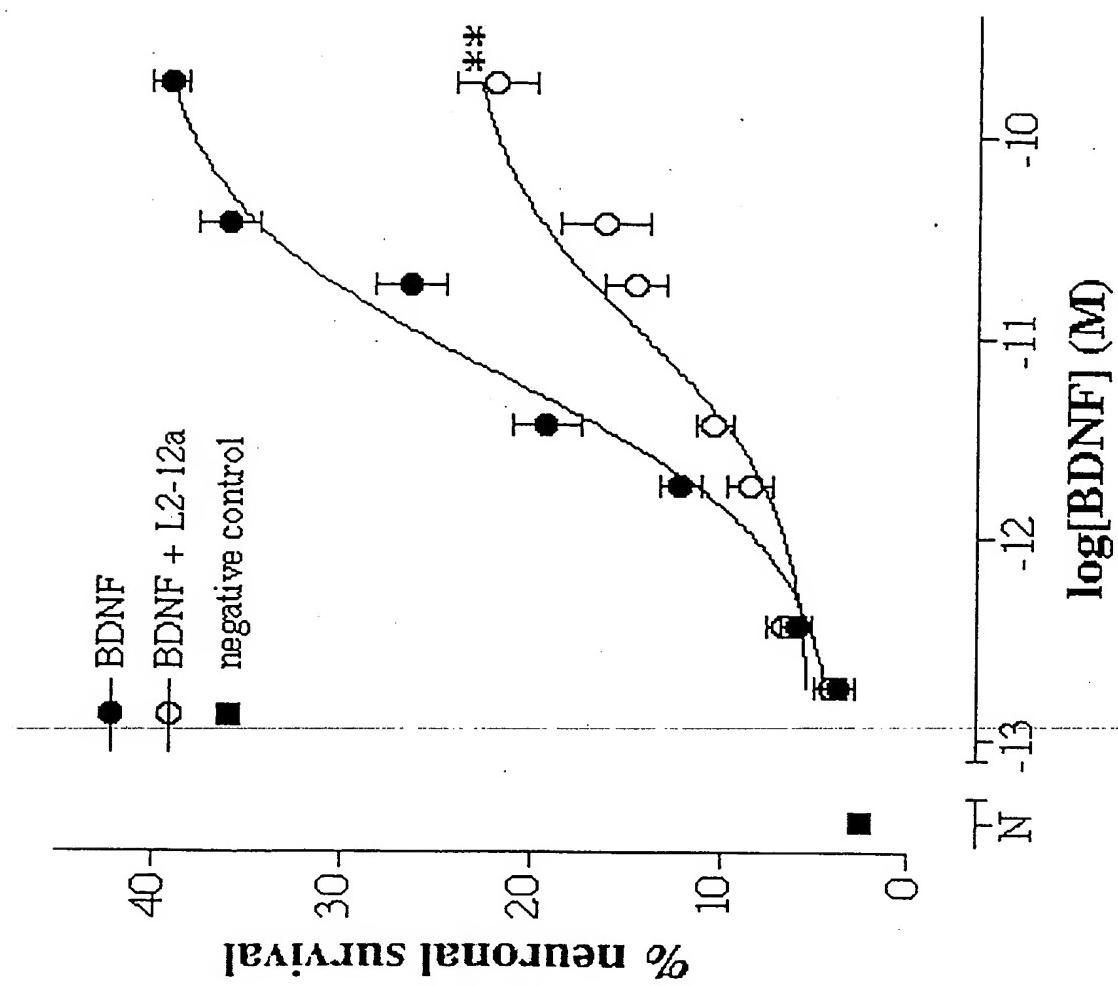
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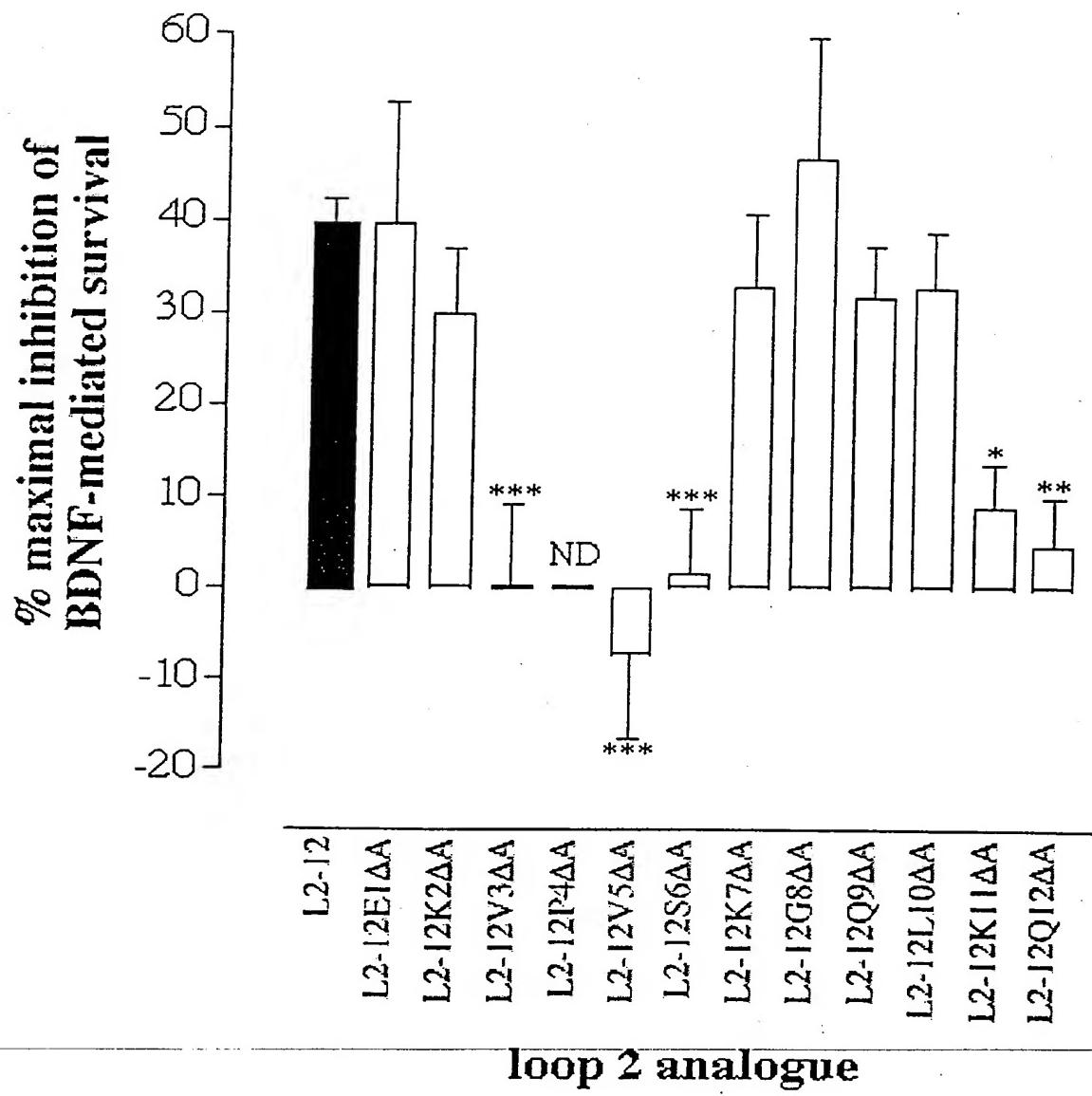
FIGURE 6

Schematic view of loop 2
region of BDNF

6.2Å

Ser .... Ser

5.4Å

Val ... . :. . Val

11.3Å

Pro . .:..:. Pro
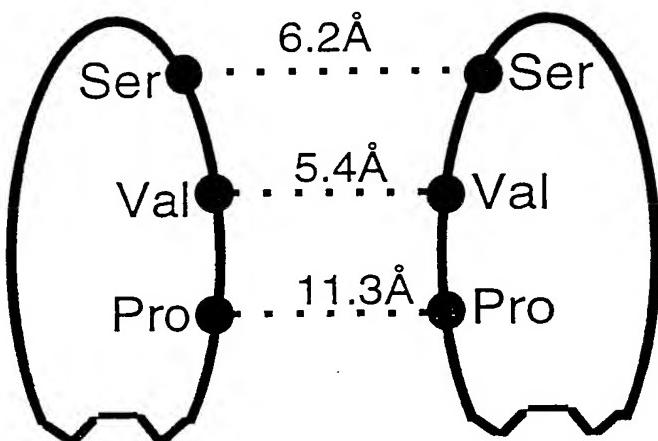
FIGURE 7A

Schematic view of disulphide bridge
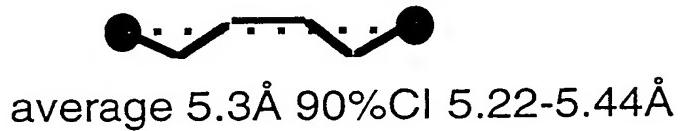
FIGURE 7B

average 5.3Å 90%CI 5.22-5.44Å

FIGURE 8

FIGURE 9

$(L28\&E+K)_2$

FIGURE 10

$(L28S4C\&E+K)_2$
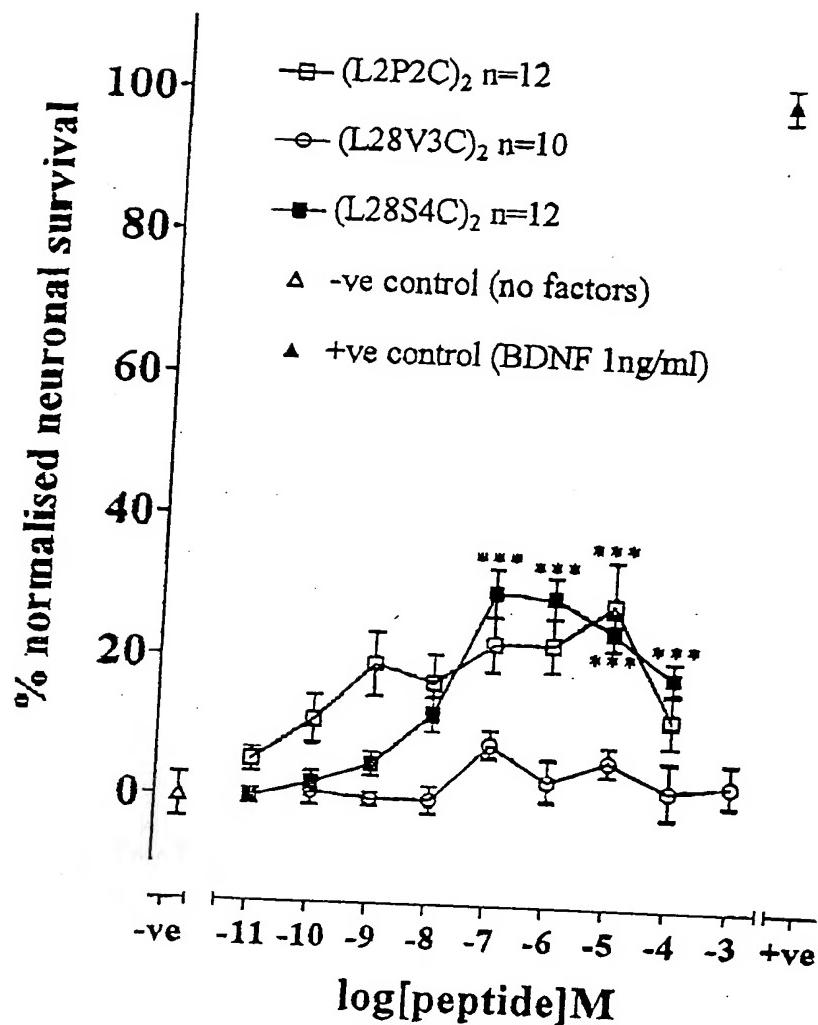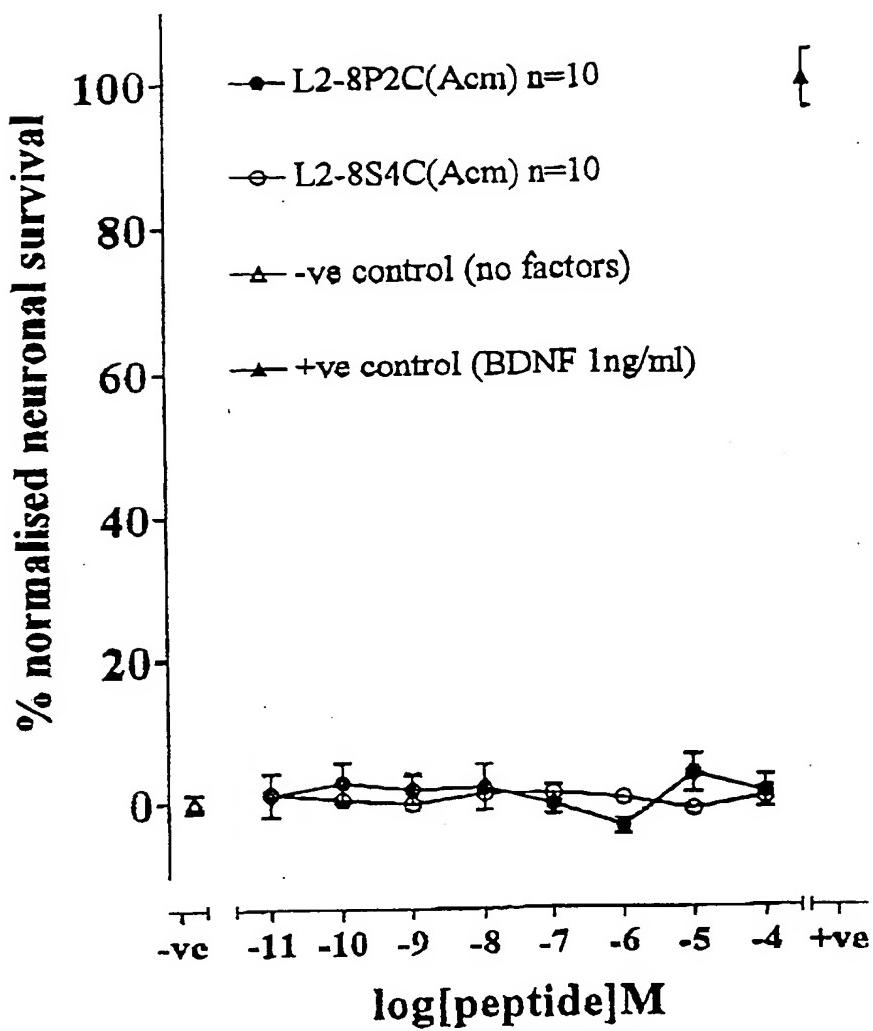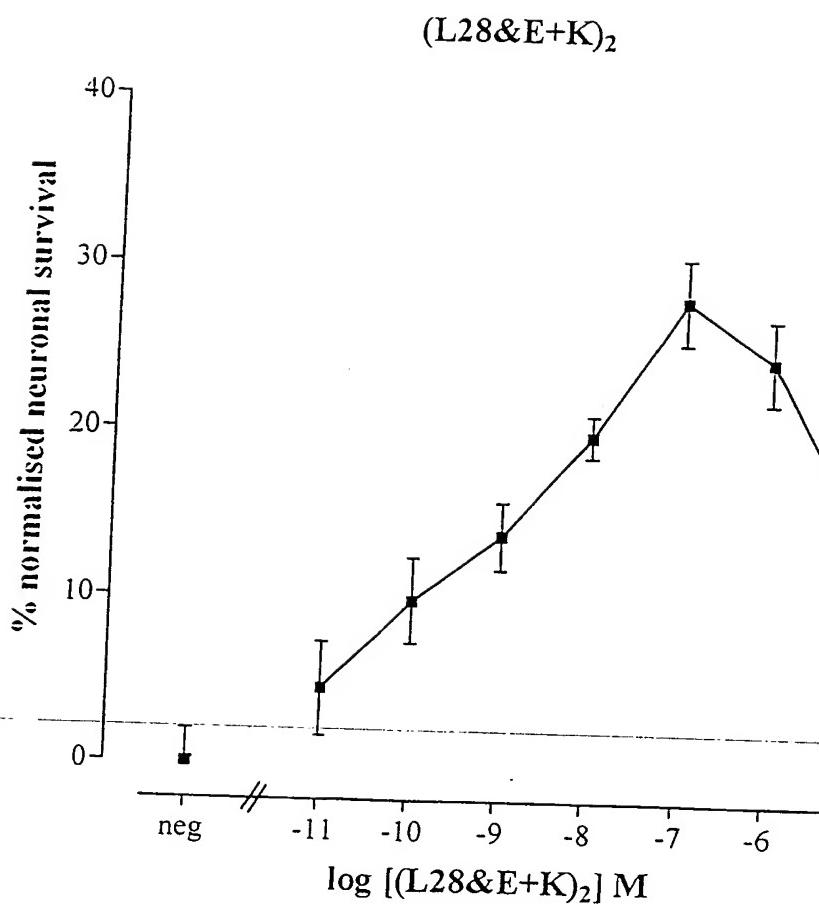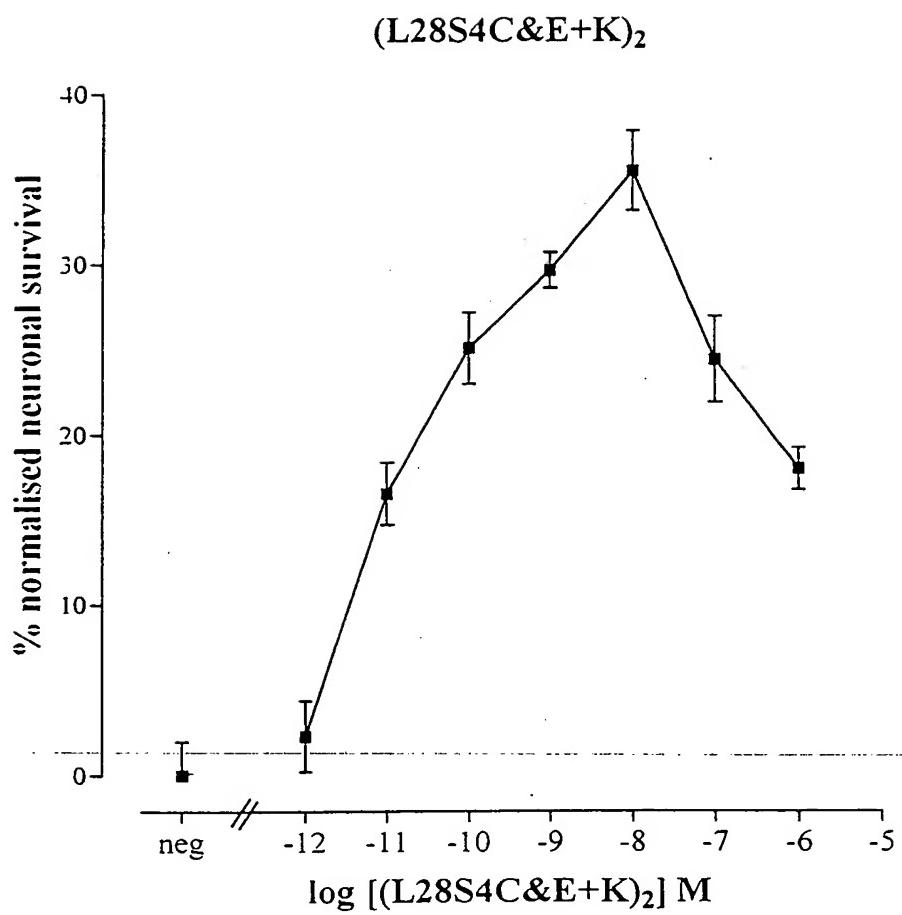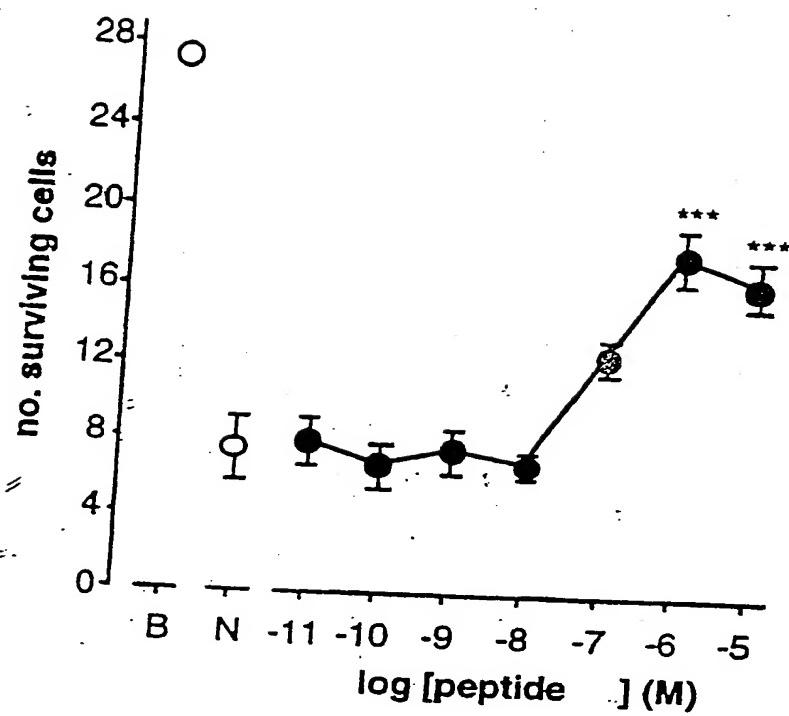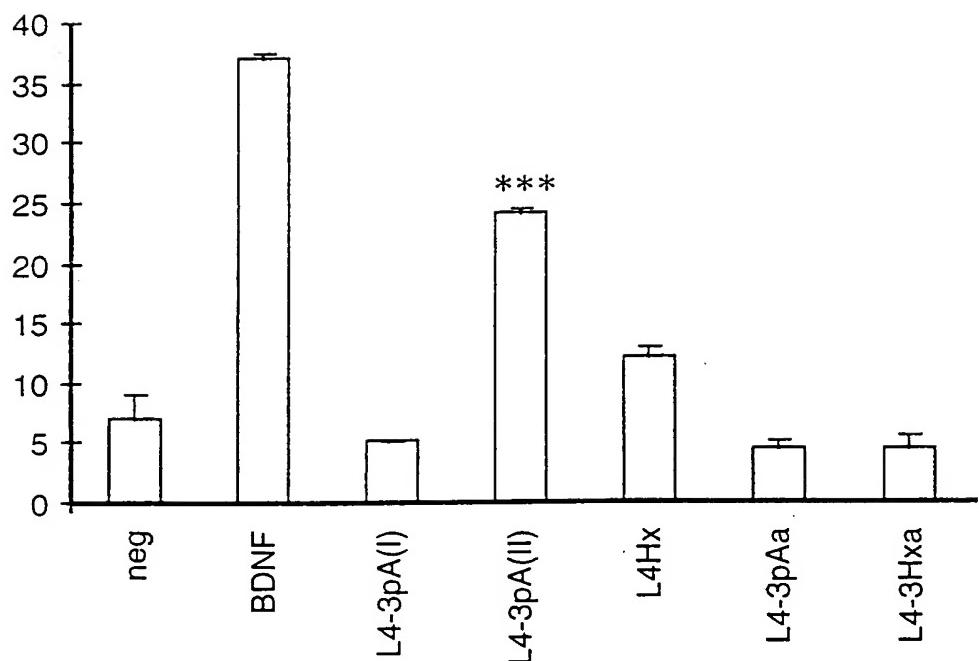
FIGURE 11

FIGURE 12

FIGURE 13
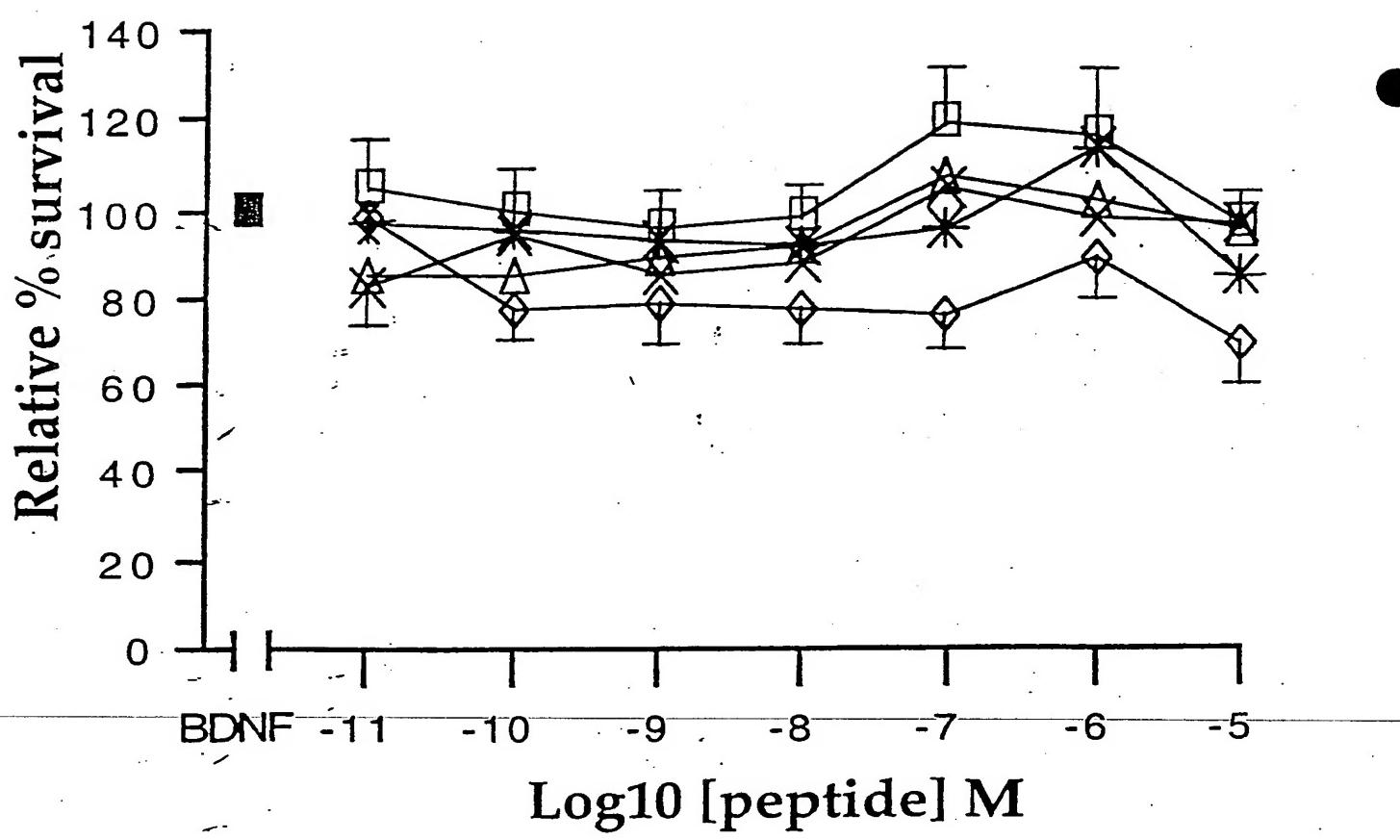
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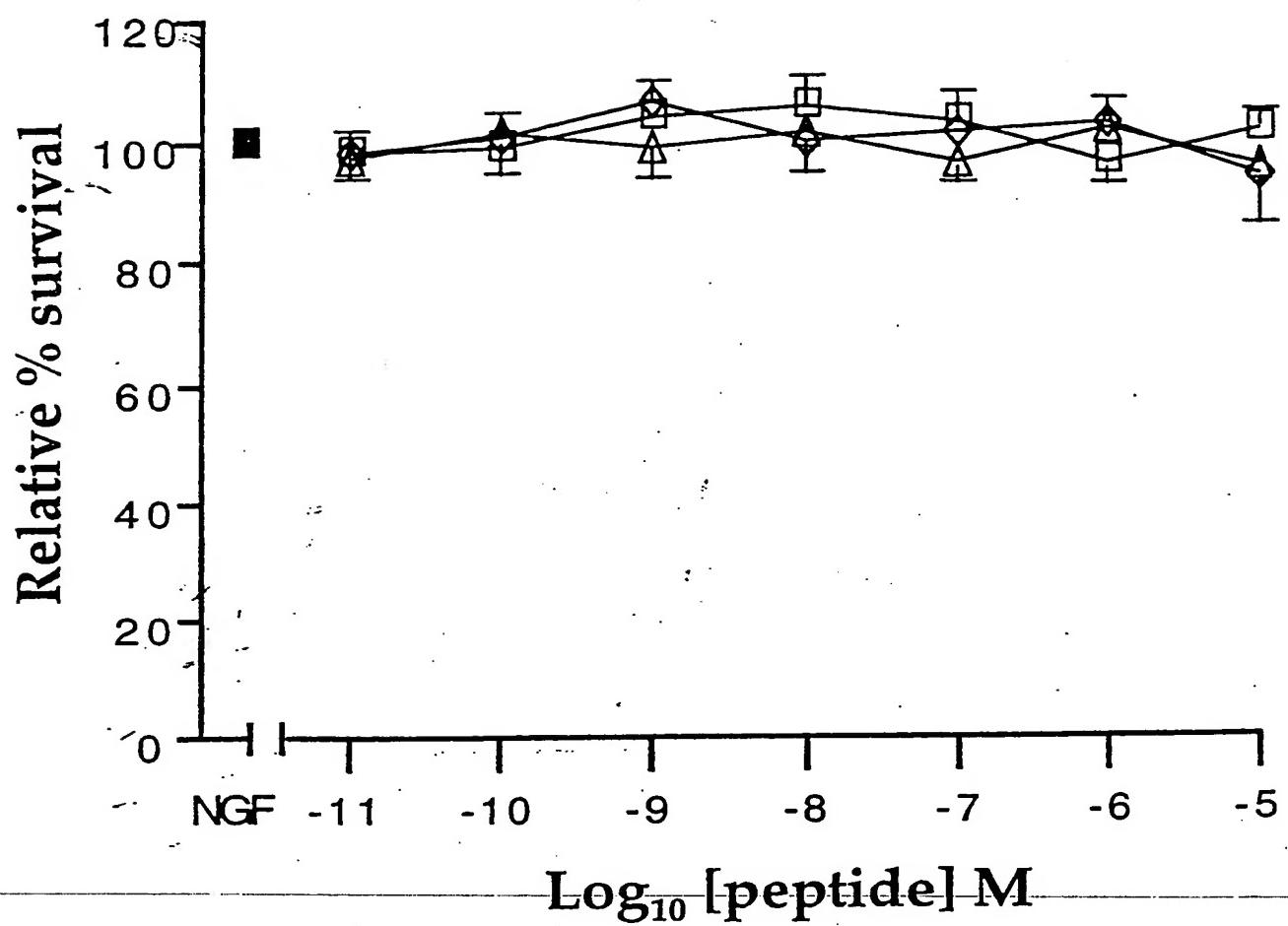
FIGURE 15

FIGURE 16

FIGURE 17

LYS+ 3 LYS+ 4

ALA 2

ARG+ 5

DPRO 1

FIGURE 18